INTERACTIONS BETWEEN FLAVONOIDS, VITAMIN C AND VITAMIN E IN PROTECTING MEMBRANES AGAINST OXIDATIVE STRESS

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ABSTRACT

Vitamin C, as a water-soluble antioxidant and vitamin E being the major lipid-soluble antioxidant, play vital roles in preventing oxidative damage. Flavonoids are a class of plant secondary metabolites well known for antioxidant activities that due to their amphiphilicity may interact with both vitamin C and vitamin E. This research investigates their cooperation with vitamin C and vitamin E on oxidative status *in vitro* using artificial liposomes and isolated rat liver mitochondrial membranes.

Vitamin C induced pro-oxidant effects in both systems and the oxidative stress was positively correlated with its concentration. The presence of vitamin E in liposomes inhibited the pro-oxidant effect of ascorbic acid, and the flavonoid quercetin inhibited this effect in vitamin E-free liposomes.

Experiments with added *tert*-butyl hydroperoxide were used to test for a possible cooperation between vitamin C, flavonoids, and vitamin E in inhibiting lipid peroxidation. While most of the conditions did not reveal cooperation, evidence for cooperation involving quercetin was observed in liposomes at the lowest level of vitamin E tested.

In measurements of vitamin E (α-tocopherol) content in mitochondria using HPLC and mass spectrometry, the combination of vitamin C and quercetin was able to synergistically regenerate the α-tocopherol in the presence of hydroperoxide while the regeneration did not happen with either vitamin C or quercetin alone. However, the recovery of vitamin E did not relate to the TBARS measurement, indicating that the α-tocopherol concentration was not directly correlated to the TBARS formation.

Based on this research, acorbate has pro-oxidative effects on both systems, which can be suppressed by quercetin but not completely by vitamin E. Among the tested flavonoids, only quercetin was observed to synergistically interact with acorbate to regenerate α-tocopherol, although under this condition the interaction did not rescue the mitochondria from oxidative damage.

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At last but not the least, to my wife Xi Lei, for your great patience and it is finally done!

DEDICATION

This work is dedicated to my grandmother, Jifang Chen, who gave me strength and still prays for me in another world.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
Asc	ascorbic acid
BHT	butylated hydroxytoluene
CEHC	carboxyethyl hydroxychroman
CL	cardiolipin
CytC	cytochrome *c*
DHA	dehydroascorbic acid
DMSO	dimethyl sulfoxide
EGTA	ethylene glycol tetraacetic acid
GLUT	glucose transporters
GSH	glutathione
GSSG	glutathione disulfide
γ-T	γ-tocopherol
HPLC	high-performance liquid chromatography
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

KPi	potassium phosphate
LDL	low-density lipoprotein
LLOQ	lower limit of quantification
LH	unsaturated lipid
LOOH	lipid hydroperoxides
Lipo	liposome
LipoE	liposome with vitamin E
MW	molecular weight
MDA	malondialdehyde
NO	nitric oxide
PC	phosphatidylcholine
PE	phosphatidylethanolamine
Quer	quercetin
ROS	reactive oxygen species
RNS	reactive nitrogen species
RDA	recommended dietary allowance
RLM	rat liver mitochondria

SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SD	standard deviation
SVCT	sodium-dependent vitamin C transporters
TBA	2-thiobarbituric acid
t-buOOH	*tert*-butyl hydroperoxide
TBARS	thiobarbituric acid reactive substances
UCACS	University Committee on Animal Care and Supply

1. INTRODUCTION

1.1. Vitamin C

In 1767, using the first recorded controlled experiment, James Lind established the scientific basis of scurvy and citrus fruits were found able to prevent scurvy. The antiscorbutic factor in citrus fruits was found in 1933 and named as L-ascorbic acid (Figure 1) by Albert Szent-Györgyi, and it was confirmed to be vitamin C. Scurvy was once believed to be exclusive to humans, which was proved wrong but still, the human is one of the few species that cannot synthesize vitamin C. The inability to synthesize vitamin C is due to lack of the enzyme, gulonolactone oxidase, which is essential in the last step of vitamin C synthesis (1).

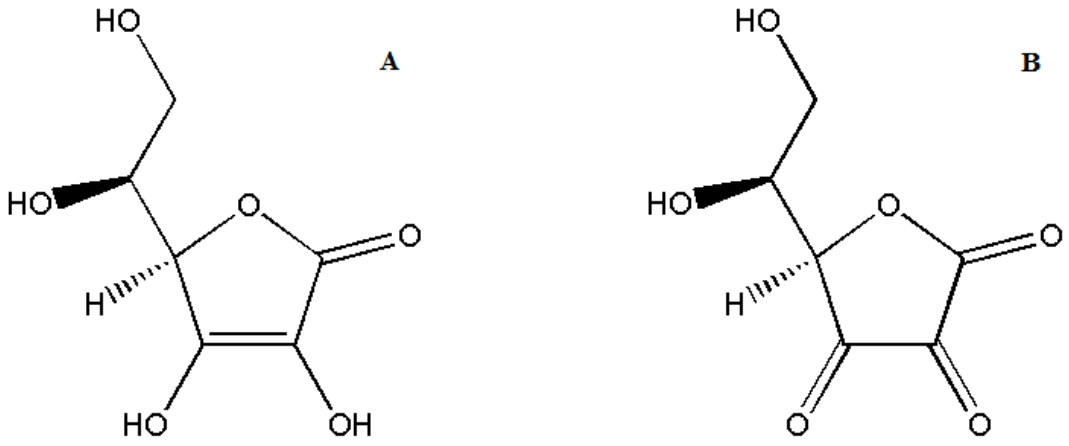


Figure 1. *Vitamin C in reduced form (**A**, L-ascorbic acid) and oxidized form (**B**, dehydroascorbic acid, DHA).*

With accumulated research on vitamin C, more of its functions were discovered. Now it is believed that it has two primary functions *in vivo*; it works as a cofactor facilitating

specific enzyme actions, notably in collagen synthesis, and as an antioxidant protecting cells from oxidative stress (2).

1.1.1 Absorption pathways

Vitamin C can be taken up into cells in both oxidized and reduced forms. The oxidized form, dehydroascorbate (DHA), may be absorbed by two pathways, either by simple diffusion or by glucose transporters (GLUT) (3). The interaction between DHA and GLUT has been studied for years (4-6). Consistent evidence suggests that DHA in some tissues shares the same transporters with glucose (5, 7). There are several glucose transporter (GLUT) isoforms interacting with DHA, differing by their substrate affinity, tissue distribution, and efficiencies (3). GLUT1 and GLUT4 are the major DHA transporters. GLUT1 mediates the transport of DHA with a similar rate to that for glucose, and GLUT4 possesses a lower rate compared to glucose. GLUT2 and GLUT5 are unable to facilitate this process, while GLUT 3 is only able to transport the reduced form of ascorbate (8).

The other cell entry pathway for DHA does not require energy and is sodium-independent (9). Once the DHA enters the cell, it is reduced to ascorbate immediately, generating a gradient of DHA across the membrane (9). Chemical reduction by glutathione (GSH) (10) and enzymatic reduction by dehydroascorbate reductase (11) are two major pathways of maintaining the DHA gradient.

The reduced form, ascorbate, is accumulated by two sodium-dependent vitamin C transporters (SVCT1 and SVCT2), which were cloned and characterized in 1999 (12). The two transporters are distributed in different locations across the body. SVCT1 is

more concentrated in epithelial cells of kidney, intestine and liver, which may account for the whole body homeostasis involving intestinal absorption and renal reabsorption, whereas SVCT2 is widespread in most tissues and participates in the general uptake of the ascorbate in most body tissues (12-14). The general cell uptake pathway is summarized in Figure 2.

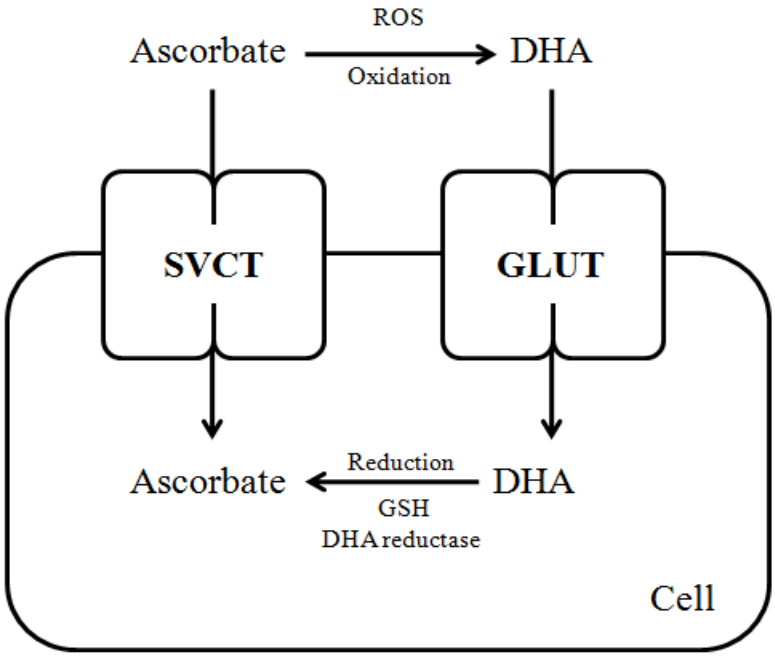


Figure 2. *Cell-uptake pathways of vitamin C (adapted from (15)).*

1.1.2 Antioxidant and pro-oxidant effects

Besides its indispensible role in the synthesis of proteins *in vivo*, vitamin C is also well-known for its ability to donate electrons and hydrogen atoms, serving as an antioxidant (16). The main target of antioxidants is free radicals, which are mainly derived from reactive oxygen species (ROS) and reactive nitrogen species (RNS). Free radicals can react with vitamin C and be reduced to a stable state. Free radicals are not always playing havoc in our system, as they can also serve as signalling molecules, or second messengers.

The reactive side of the free radicals can lead to tissue damage and ultimately, the aging process. The chemical nature of free radicals is that their unpaired electrons make them highly reactive and they tend to abstract hydrogen atoms from adjacent molecules. There are two major properties making vitamin C an ideal antioxidant, the low reduction potentials of both ascorbate and ascorbyl radical (17), and the stability plus low reactivity of the intermediate radical (18). Also, ascorbate is present at relatively high concentration (1-3 mM) in cells (2). The oxidation-reduction cycle of ascorbate is shown in Figure 3. When meeting a free radical, ascorbate first donates one hydrogen atom, forming a semidehydroascorbic acid radical. Abstraction of a second hydrogen atom leads to the formation of dehydroascorbate which can be reduced to ascorbic acid by dehydroascorbic acid reductase using glutathione. Alternatively, two intermediate radicals can react with each other, yielding one oxidized and one reduced form of vitamin C (2).

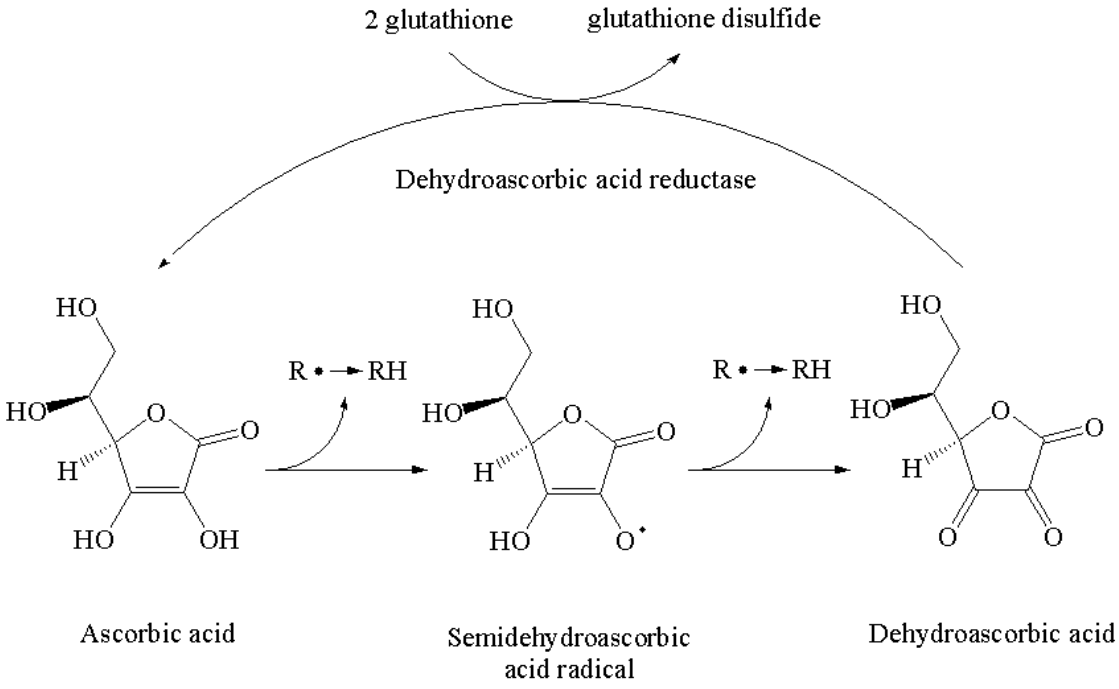


Figure 3. *Cycle of vitamin C as an antioxidant (Adapted from S.S. Gropper (2)).*

Like a double-edged sword, vitamin C can also act as a pro-oxidant, depending on the environment and conditions where the molecule is active (19-21). The mixtures of iron salts and ascorbate have been used for decades to stimulate free-radical oxidation of lipids (lipid peroxidation) *in vitro*. Bucher et al (22) established a Fenton reaction-like model of ascorbate and transition metals in initiating the lipid peroxidation. Acorbate can stimulate free-radical reactions under certain circumstances by reducing Fe (III) to Fe (II). Fe (II) reacts with hydrogen peroxide to give the highly reactive hydroxyl radical ($OH^{\bullet}$). Other researchers also reported that ascorbate can be a pro-oxidant due to its ability to reduce transition metals (17). Lee et al. reported in *Science* (23) that vitamin C, even without the presence of catalytic iron, promoted the decomposition of lipid hydroperoxide to DNA-reactive electrophiles *in vitro*, which could lead to serious DNA damage.

In *in vivo* studies, the pro-oxidant effect of vitamin C is still not fully understood and generates debate (24-35). In a prominent study reported in *Nature* (36), 500 mg/day supplements in healthy individuals showed an increase in lymphocyte DNA oxidative damage. In another study with human volunteers, Rehman et al (28) reported that the co-supplementation of iron (14 mg/day ferrous sulphate) and vitamin C (60 mg/day or 260 mg/day) caused a significant rise in DNA damage (100% higher than baseline levels), that returned to normal after a longer period of supplementation. Also, consistent with the observation *in vitro* that ascorbate decomposes lipid hydroperoxides to genotoxic aldehydes (23), 400 mg/day supplementation of vitamin C to humans produced an acute increase in blood levels of these aldehydes by 70% (37). However, the same amount of ascorbate did not affect the DNA damage level in peripheral blood mononuclear cells

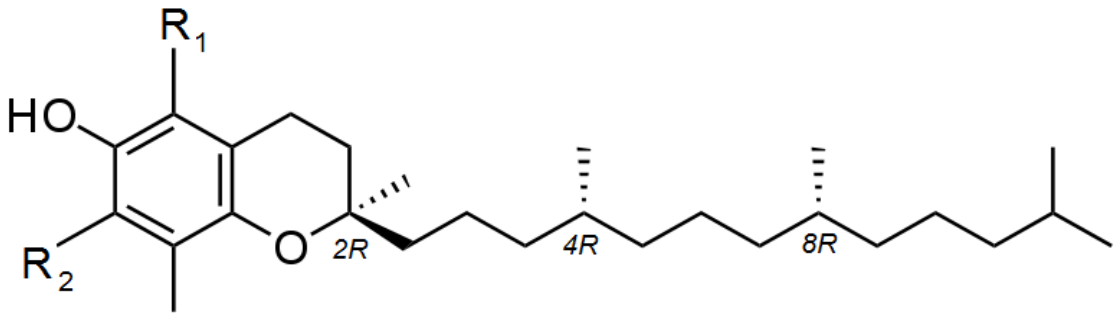
(38). When supplemented by 500 mg/day, smoking people (who have decreased plasma ascorbic acid levels) were found to have an increase in plasma TBARS after two months of supplementation (39). However, other studies showed that vitamin C supplementation inhibited rather than promoted iron-dependent oxidative damage. For example, vitamin C deficient guinea pigs loaded with iron experienced ameliorated lipid oxidation when supplemented with vitamin C, while low vitamin C plus high iron status caused additional pathophysiological changes (32).

Overall, the data on whether ascorbate supplementation alone induces oxidative stress *in vivo* is inconsistent and conflicting. One possibility for differences between studies might be in the levels of other dietary antioxidants such as vitamin E or flavonoids. In this thesis I investigated *in vitro* the abilities of α-tocopherol and different flavonoids to influence the pro-oxidant effect of ascorbate on lipid peroxidation, as well as to cooperate with ascorbate as antioxidants.

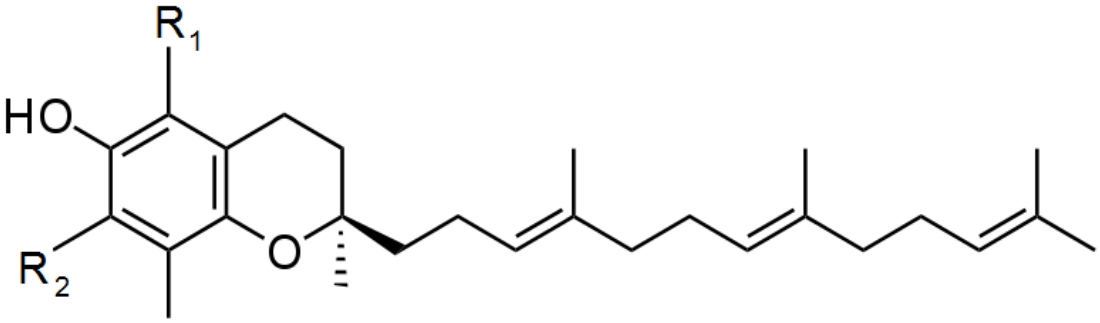
1.2. Vitamin E

In 1922, Evans and Bishop discovered a factor X in unprocessed food which was indispensible for reproduction of the rats (40). Later, with much effort invested in its research, this factor named vitamin E has been known for many important roles in sustaining our health, ranging from antioxidant property to its influence in signalling cascades. Vitamin E now is defined as the collective name for a set of 8 related α-, β-, γ-, and δ-tocopherols and tocotrienols (41), which are all fat-soluble antioxidative vitamins. The tocotrienols have unsaturated side chains, which are saturated in tocopherols instead. The tocopherols differ by the location and number of the methyl groups on the chromanol ring (Table 1), and their counterparts in tocotrienols share the similar structure on the

chromanol rings (Figure 4). Each of the tocopherols is composed of eight stereoisomers when derived from nonchiral chemical synthesis (termed all-*rac*- or *dl*-), while all naturally occurring tocopherols have the same molecular configuration [*RRR*, *d*-tocopherols or (+)-tocopherols] in their phytyl groups (42). Although sharing many crucial properties, each form of vitamin E has its own unique properties, with alpha- and gamma- tocopherol attracting the most research interest.



Tocopherols



Tocotrienols

Figure 4. *Tocopherols and tocotrienols*

Table 1. *Different groups in tocopherols and tocotrienols. Tocopherols are shown in their naturally occurring RRR-configuration.*

	R_1	R_2
α- tocopherol/tocotrienols	CH_3	CH_3
β- tocopherol/tocotrienols	CH_3	H
γ- tocopherol/tocotrienols	H	CH_3
δ- tocopherol/trienols	H	H

1.2.1 Vitamin E in diet

As humans are incapable of synthesizing vitamin E, its level in our body is maintained by dietary intake. Foods made from vegetable oils are good sources of the vitamin E family, especially the ones made with full-fat content, such as salad dressings, margarine and peanut butter (43).

Alpha-tocopherol is the predominant form of vitamin E, as its concentration in human plasma and tissues was higher than other homologues (44, 45). It can be found in food either from plant or animal and is the primary form of vitamin E in supplements. The Recommended Dietary Allowance (RDA) for vitamin E before 2000 was based on "α-tocopherol equivalents" (46), while in the 2000 edition, the RDA for vitamin E can only be met by α-tocopherol and the activity of synthetic (*dl*) α-tocopherol is specified as half of the natural (*d*) α-tocopherol (47). Vegetable oils, including canola, olive, sunflower, safflower and cottonseed, are great sources of α-tocopherol. It is also stored in plants leaves and other green parts containing chloroplasts (43). Besides the plant sources, it is also in animal originated foods, especially in fat tissues (43).

Gamma-tocopherol is the principal vitamin E form in the US diet, being about 2.5 times more abundant in food than α-tocopherol (48). Its source is principally vegetable oils (49). This form of vitamin E is more common in plant than in animal sources. In some vegetables oils, such as corn and soybean oil, its concentration is significantly higher than that of α-tocopherol (43). Because of the widespread use of these plant products, gamma-tocopherol represents 70% of the vitamin E consumed in the typical US diet (49).

Even though γ-tocopherol is the predominant class of dietary vitamin E in North America diets, α-tocopherol is the most relevant vitamin E form for human physiology, accounting for the majority of this vitamin present in mammalian tissues (50). The main sources of beta- and delta-tocopherols are nonchloroplast regions of plants and tocotrienols are usually found in cereal grains and legumes (43). Other classes of vitamin E are less studied either due to their insignificant amounts in the diet and tissues or the biological function of which is not different from the two major classes.

1.2.2 Lipid peroxidation and vitamin E

Vitamin E, as a powerful peroxyl radical scavenger, performs its anti-oxidative property in a chain-breaking mechanism to prevent the lipid peroxidation chain reaction in membranes and in plasma lipoproteins (51). Lipid peroxidation is a typical radical chain reaction. This reaction is initiated by reactive oxygen species (ROS), produced as a byproduct of oxygen metabolism and plays essential roles in cell signaling and homeostasis (52). ROS deplete hydrogen atoms from bis-allylic C–H bonds in unsaturated lipids, forming lipid radicals (53). Lipid radicals continue to react with molecular oxygen yielding lipid peroxyl radicals, and initiate the propagation reaction if not reduced by vitamin E (Figure 5). The peroxyl radicals are not stable and tend to

deplete hydrogen atoms from other free lipids, forming different fatty acid radicals and lipid peroxides.

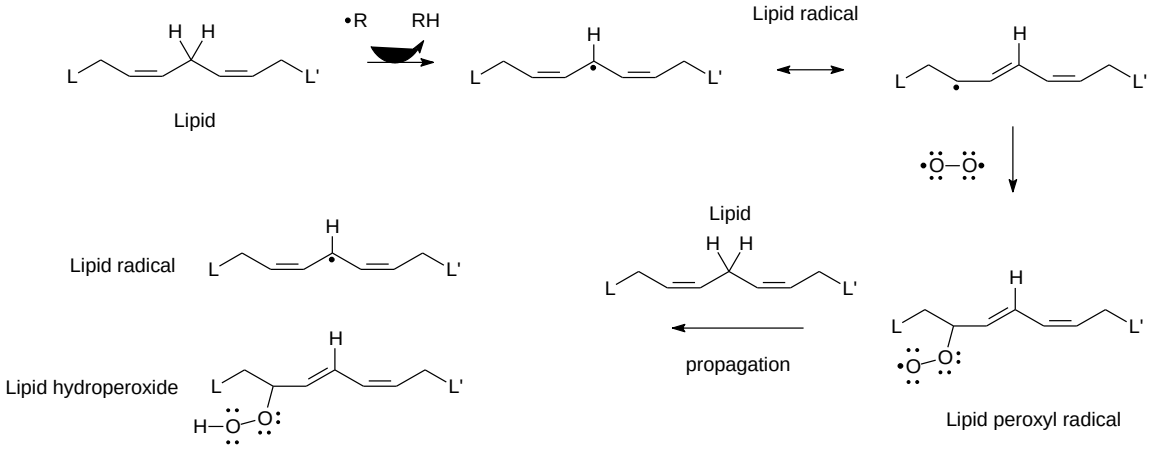


Figure 5. *Lipid peroxidation chain reaction.*

Vitamin E composes a tiny portion of the membrane and cannot compete with unsaturated lipids or oxygen in the non-specific reactions with lipid radicals to prevent the propagation. However, peroxyl radicals react 1000 times faster with vitamin E than with polyunsaturated fatty acids (54). This specific reaction reduces peroxyl radical to corresponding lipid hydroperoxides and oxidizes vitamin E to radicals (Figure 6), which are relatively stable and do not react with free oxygen. The vitamin E radicals react with other hydrogen donors (eg. vitamin C), returning vitamin E to the reduced state (43). If the radicals are not reduced, it can be further oxidized by peroxyl radicals to tocopherol quinones (55) and other oxidation products (56). Photo-oxidation can also generate other products such as dihydroxy dimer, spirodimer, R-tocopherolquinone 2,3-epoxide, R-tocopherolquinone 5,6-epoxide, and 8a-(hydroperoxy)epoxytocopherones (57). Although all 8 homologues show antioxidant properties (58), the α-isomers are the most active scavengers among them (59).

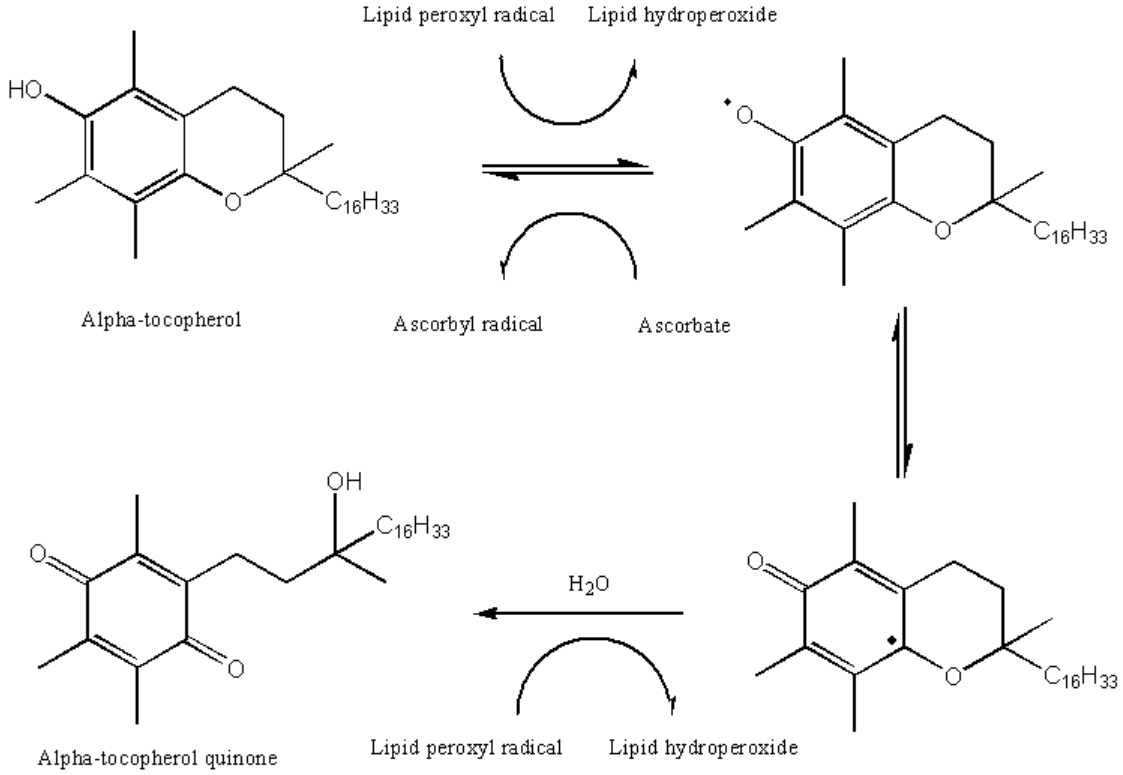


Figure 6. *Antioxidant effect of α-tocopherol.*

1.2.3 Metabolism of vitamin E *in vivo*

When vitamin E is absorbed excessively *in vivo*, it will be excreted instead of accumulated like the other fat-soluble vitamins (43). Vitamin E isomers are initially ω-oxidized by cytochrome P450s (CYPs), followed by several steps of β-oxidation, and then conjugated and excreted in urine (60) or bile (61). The excreted metabolites are tail-shortened and carboxylated (Figure 7), with the same double-ring head structure derived from either tocopherols or tocotrienols (62). Delta- carboxyethyl hydroxychroman (δ-

CEHC) is the first to be reported among the carboxylated metabolites (63), while α-CEHC and γ-CEHC were later discovered and shown to be present in plasma (64).

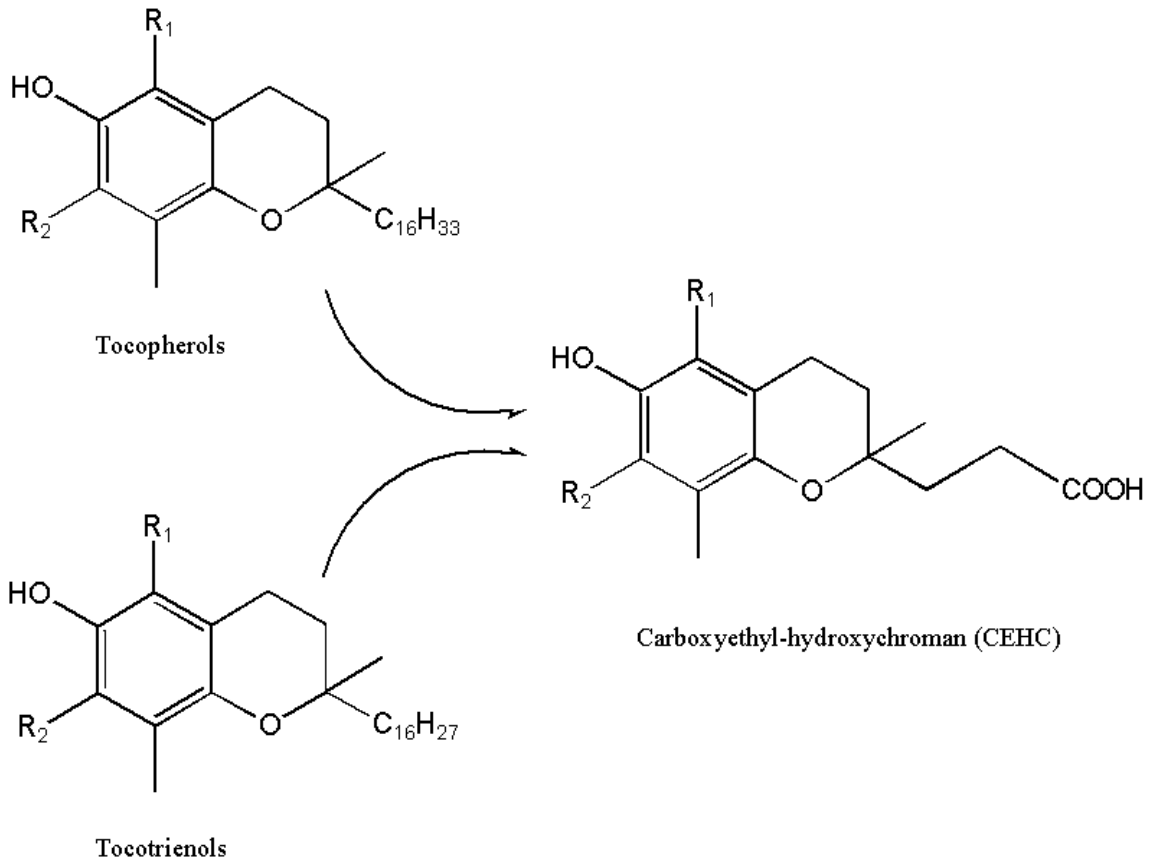


Figure 7. *Metabolism of tocopherols and their corresponding tocotrienols to carboxyethyl-hydroxychromans (CEHC).* The tocopherols and tocotrienols are hydroxylated by cytochrome P450s (CYPs) and further carboxylated, followed by several steps of β-oxidation yielding the corresponding CEHC (65).

1.3. Flavonoids

Flavonoids are a class of plant secondary metabolites and are common in our daily diets (Figure 8). In plants, they perform multi-functions as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, and light screens (66). Over 4,000

types of flavonoids have been identified in the vascular plants and they vary in quantity and type due to difference in plant growth, habitats and maturity. Depending on the oxidation level on the heterocycle, flavonoids can be divided into several categories: flavones, flavonols, isoflavones, anthocyanins, flavanols, proanthocyanidins and flavanones (67). All of these different classes share a similar triple-ring structure (Figure 8, quercetin as an example), with 2 aromatic rings on the sides of a heterocyclic ring.

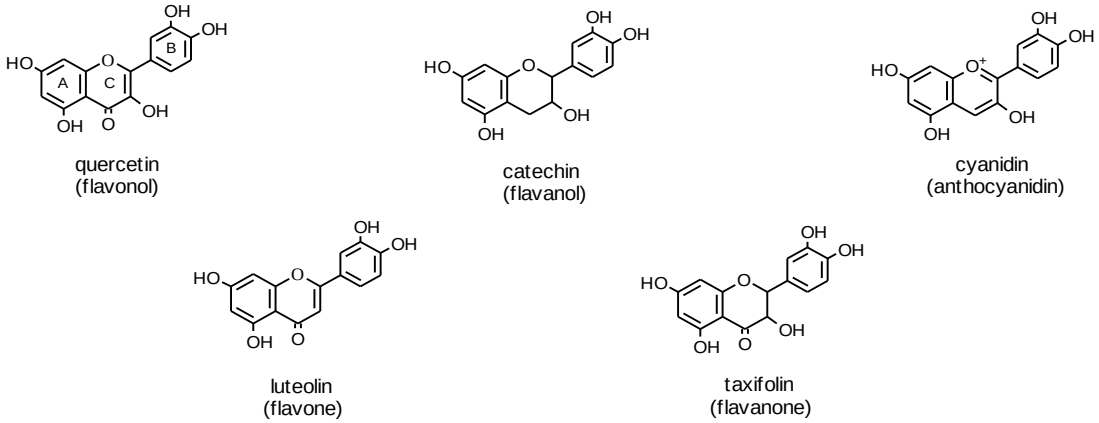


Figure 8. *Flavonoid classes (in parentheses) and examples.*

The flavones and flavanones are found in citrus fruits, while the anthocyanins are pigments of berries and grape skins. The catechins are a well known flavonoid type of teas, while quercetin is a major dietary flavonoid that can be found (primarily as glycosides) in black and green tea, apples and onions (68).

As the plasma levels of flavonoids are directly related to human daily dietary intake and their compositions in various plants differ greatly (69-71), it is urgent to evaluate the flavonoid sources in food as well as their health benefits.

1.3.1 Absorption and interaction with lipid membranes

Prior to absorption, dietary flavonoids are released by chewing, and reacting with digestive juices in the gastrointestinal tract and the microorganisms of the colon. Predominantly present as glycoside conjugates, they can be absorbed intact in only a small portion in the small intestine (72). A large portion of the flavonoids undergoes hydrolysis by lactase phlorizin hydrolase, yielding the aglycone in the brush-border of the small intestine epithelial cells (73).

Before entering the blood stream, the aglycones undergo phase II metabolism forming sulfate, glucuronide and/or methylated metabolites through the respective enzymes. Some of the metabolites may efflux back into the lumen of the small intestine. Once merging into the bloodstream, metabolites rapidly reach the liver, where they can be subjected to further phase II metabolism and conversions, and enterohepatic recirculation may result in some recycling back to the small intestine through bile excretion (72).

The structural characteristics of flavonoids determine their interaction with the lipid membrane. The intensity of this interaction at the interface of phospholipid membranes correlates positively with the number of polyphenol hydroxyl groups while the influence on membrane structure correlates to the hydrophobicity of the molecules (74). However, increasing hydroxyl moieties in one molecule results in increasing hydrophilic solubility and reduces its ability to scavenge radicals within the membrane (75). More properties, such as the three dimensional structure (76) and methylation of hydroxyl groups (77) will also affect their interaction with lipid bilayers, and this latter characteristic of the flavonoids is considered as a dominating factor in reacting with membranes (77).

1.3.2 Antioxidant property of flavonoids

Previous studies have demonstrated that flavonoids exhibit multiple biological activities, such as anti-allergenic (78), anti-viral (79), anti-inflammatory (80), and vasodilating actions (81). However, most interest has been focused on their antioxidant activity, which is due to their ability to scavenge free radicals and to reduce their formation. The general chemical features of flavonoids in scavenging free radicals are: 1, the phenol group which can donate one electron to reduce radicals; and 2, the aromatic structure which stabilizes the aroxyl radical by resonance (82).

According to Halliwell and Gutteridge (83), to be a successful antioxidant, the substance should include functions as follows: 1, suppress ROS formation either by inhibition of enzymes or chelating trace elements involved in free radical production; 2, scavenge ROS; and 3, upregulate or protect antioxidant defenses.

Flavonoids have been confirmed to perform all of the criteria described above. Flavonoids inhibit enzymes such as protein kinase C (84), lipoxygenase (85), microsomal monooxygenase (86), mitochondrial succinoxidase, and NADH oxidase (87), which are all involved in ROS generation. In addition, a number of flavonoids are able to chelate trace metals involved in free radical production. Free copper and iron are potential activators of ROS formation. Metal-catalyzed oxidation and reduction of H_2O_2 (the Fenton reaction) can result in highly aggressive hydroxyl radical generation. Copper can also mediate LDL oxidation (88). In regard to their radical-scavenging properties, most research proved that flavonoids scavenge superoxide anion radicals (89, 90), as well as peroxyl (91), hydroxyl (92), alkoxyl (82), and nitric oxide (93) radicals. Concerning all these results, it becomes readily apparent that flavonoids are highly effective scavengers

of most types of oxidizing species. As for the mechanism of their antioxidant effects, many studies have been performed to discover the relationship between their structure and the ROS scavenging activity. The hypothesis established by Bors et al (82) has been considered as the most convincing theory. According to Bors, the major determinants for radical scavenging capacity are, first, the presence of a catechol group in ring B, whose electron donating properties are strong and is a radical target, and second, a 2,3- double bond conjugated with the 4-oxo group, which is responsible for electron delocalization.

The antioxidant activity of flavonoids is not restricted to the aqueous phase, but they also participate in the lipid phase by their dual-phase solubility. Concerning the flavonoid-ascorbate synergistic action, Bandy and Bechara reported (94) that, using synthesized liposomes *in vitro*, some flavonoids (epicatechin and quercetin but not rutin) allow them to transfer electrons from ascorbate to a hydrophobic environment and thereby mediate antioxidant protection to membrane-bound cytochrome *c*. Also in two other studies, flavonoid-ascorbate combination was observed to have better performance as antioxidant than ascorbate did alone, where ascorbate even gave pro-oxidant activity in the absence of flavonoids in erythrocytes and cutaneous tissue-associated cells (95, 96).

However, flavonoids are not perfect antioxidants as they have been reported to produce hydrogen peroxide in common tissue culture media at a concentration of 100 μM (97). In another study, 24 hours administration of apple juice extract (100 μM flavonoid equivalency) on human colon adenocarcinoma cells (HT29) generated hydrogen peroxide as well, while its concentration was less than that generated by media treated with the extract (98). The author also pointed out that the unphysiologically high polyphenol concentrations were not expected *in vivo* (98).

1.4. Interactions between vitamin C, vitamin E and flavonoids

In comparing the three types of antioxidants mentioned so far, their standard reduction potentials are summarized in Table 2 below.

Table 2. *Thermodynamic aspects of different free radical scavengers.*

Compound	Reduction reactions (addition of electron)	$E^{o}_{(R\bullet/RH)}$ (mV)	Reference
Lipid hydroperoxide	$LOO^{\bullet} \rightarrow LOOH^{*}$	1000	(54)
Polyunsaturated lipid	$L^{\bullet} \rightarrow LH$	600	(54)
Ascorbic acid	$A^{\bullet} \rightarrow AH$	282	(99)
α-tocopherol	$\alpha\text{-}T^{\bullet} \rightarrow \alpha\text{-}TH$	500	(100)
Quercetin	$Q^{\bullet} \rightarrow QH$	330	(101)
Rutin	$Rut^{\bullet} \rightarrow RutH$	600	(101)
Catechin	$Cat^{\bullet} \rightarrow CatH$	570	(102)
Cyanidin	$Cy^{\bullet} \rightarrow CyH$	No source	

* RH = reduced organic compound (R• = corresponding radicals); LOOH = lipid peroxide; AH = ascorbate; α-TH = α-tocopherol; QH = quercetin; RutH = rutin; Cat = catechin; CyH = cyanidin.

Most free radicals undergo simple first- and second-order reactions, and therefore the tendency of the basic electron transfer reactions is predictable. The standard reduction potential (E^{o}) represents the tendency of a chemical species to acquire electrons and thereby be reduced. We can predict the electron flow by using this potential and determine whether certain reactions can occur. Electrons are acquired from the molecules with lower potential and transferred to the molecules with the higher potential. The

potentials of flavonoids in this research are in the same range as the other two well-known antioxidants (vitamin C & E). All of the listed antioxidants have a lower reduction potential compared to the propagation step in lipid peroxidation. Therefore, theoretically they can all react with the lipid peroxyl radical and prevent the oxidative damage on lipids.

Being the major lipid soluble antioxidant, vitamin E plays a vital role in preventing oxidative damage to lipids. Once oxidized to the tocopheroxyl radical, vitamin E is believed to be regenerated by ascorbate, which was first shown *in vitro* (54, 103-105) and then confirmed *in vivo,* in healthy humans (106) and smokers (107). However, there has been some debate about the occurrence of the interaction *in vivo* (108, 109), and in a previous pilot experiment in our laboratory, the rats supplemented with vitamin C on a vitamin E deficient diet did not show protection from increased susceptibility to erythrocyte hemolysis (unpublished results) as might be expected if vitamin C could efficiently regenerate vitamin E.

Another question is what factors may help protect against the pro-oxidant activities of vitamin C. When vitamin E was sufficient in rats, increased oxidative stress after vitamin C supplementation was not observed (except non-significantly in liver) using current methods (TBARS, protein thiols, GSH and GSSG levels) (110). In another previous study, liver lipid peroxidation in rats supplemented with vitamin C was only observed at low levels of dietary vitamin E (111). Combining these previous results, vitamin E appears to be a predominant factor in protecting against pro-oxidant activities of vitamin C. However it did not completely protect against ascorbate-induced lipid peroxidation of liver homogenate, or of isolated mitochondria *in vitro* (110).

Previous results in our laboratory suggested that flavonoids may play a role in protecting from pro-oxidative activities of ascorbate in certain cellular compartments or at low levels of vitamin E. Using isolated mitochondria with a normal concentration of vitamin E, added flavonoid was able to completely prevent ascorbate-induced TBARS formation *in vitro* (unpublished results). Feeding studies also suggested that flavonoids (with adequate dietary vitamin E) inhibit a vitamin C-induced increase in the susceptibility of liver homogenate to *in vitro* peroxidation (110).

Due to their amphiphilic solubility, we hypothesize that flavonoids would facilitate the vitamin E regeneration by ascorbate as a bridge between these antioxidants in two phases. In support of this hypothesis, flavonoids alone have previously been found to spare vitamin E *in vitro* (112, 113) and *in vivo* (111).

2. HYPOTHESES AND OBJECTIVES

2.1. Hypotheses

The overall hypothesis is that flavonoids, vitamin E and ascorbate interact synergistically with each other in preventing oxidative stress. It can be divided into two parts:

1) flavonoids and vitamin E reduce the pro-oxidant effects of vitamin C; and

2) flavonoids and vitamin C cooperate to regenerate vitamin E and inhibit oxidative damage to membranes.

2.2. Objectives

The research addressed four main questions:

1) whether the presence of vitamin E in liposomes would reverse the role of vitamin C as a pro-oxidant;

2) the extent to which different flavonoids (quercetin, rutin, catechin and cyanidin) can inhibit liposomal lipid peroxidation induced by vitamin C in the absence of vitamin E;

3) the extent to which the different flavonoids cooperate with vitamins C and E to inhibit lipid peroxidation of liposomes and rat liver mitochondria synergistically;

4) the extent to which the different flavonoids plus ascorbate work synergistically to regenerate and thereby spare vitamin E in rat liver mitochondria.

3. MATERIALS AND METHODS

3.1. Experimental design

The ability of flavonoids and α-tocopherol to inhibit the pro-oxidant and facilitate the antioxidant activities of ascorbate were studied *in vitro* using liposomes and mitochondrial membranes. Liposomes were used as a model membrane system where the α-tocopherol content could be manipulated. The three types of lipid (phosphatidylethanolamine, cardiolipin, phosphatidylcholine) selected in the liposome synthesis are the major components of mitochondrial membranes (114), which form a relatively comparable model with mitochondria. Mitochondria (from rat liver) were studied as an intact biologically-relevant membrane system where the interactions of ascorbate, flavonoids and vitamin E may be important to protection against oxidative stress. Mitochondria are a primary site of redox reactions in cells, which makes them susceptible to oxidative damage. With both systems, TBARS was used as an index of oxidative damage. With mitochondria, the endogenous alpha- and gamma-tocopherol contents were measured by HPLC and mass spectrometry.

The ability of different flavonoids to inhibit the pro-oxidant effect of ascorbate were studied in both membrane systems, and the ability of α-tocopherol to inhibit was studied with the liposomes. The pro-oxidant effects of ascorbate were studied in the absence of added metals, so only involved endogenous metals (such as in cytochrome *c* and other metalloproteins).

The ability of ascorbate, flavonoids and α-tocopherol to cooperate as antioxidants was studied in both membrane systems using *tert*-butyl hydroperoxide as the oxidant. The cooperative ability to regenerate or preserve α-tocopherol was studied with the mitochondrial membranes (as the more biologically complete system).

The flavonoids chosen for study were quercetin and rutin (flavonols), (+)-catechin (a flavanol) and cyanidin (an anthocyanin). These are all 2'3'-dihydroxy flavonoids from different classes, with rutin being a representative glycoside. A previous study (94) identified quercetin and catechin as flavonoids with the potential to cooperate with ascorbate to protect membranes, and rutin as a flavonoid that was unlikely to cooperate in this activity.

The concentrations of ascorbate that were used (200 and 400 μM) are those that can be achieved in blood with supplementation (100 and 250 μM) (115), and near that normally in cells (1-2 mM) (2). The concentration of flavonoids used (50 μM) was within the range (23-107 μM) observed in plasma of rats supplemented with quercetin (116). The concentration of flavonoid used therefore is lower than that of ascorbate, consistent with the flavonoid serving as a redox intermediate in the antioxidant chain.

3.2. Materials

All the materials used in the research are listed in the following table, and their names presented in this report are in the left column. All aqueous solutions were made with water that was first filtered and distilled and then purified with a MilliQ 18MΩ purification system.

Table 3. *List of materials used in the research (including abbreviations).*

Name /Abbreviation	**Full description**
PC	phosphatidylcholine, Sigma XIII E, 100mg/ml, P42791
PE	phosphatidylethanolamine, Sigma III egg yolk 125mg/ml in $CHCl_3$ P7943
CL	cardiolipin, Sigma, 4.7mg/ml, C1649
Vitamin E	approx. 670 mg d-α-tocopherol per gram. The non-α content is 5-20 mg/g; remainder is soybean oil, Sigma, T3634
α-T	dl-*all-rac*-α-tocopherol, Sigma, synthetic, ≥96%, (HPLC), T3251
γ-T	(R,R,R)-γ-tocopherol, Sigma, ≥96%, (HPLC), T1782
Quer	quercetin dihydrate, Sigma, Q0125
Catechin	(+)-catechin hydrate, Sigma, C1251
TBA	2-thiobarbituric acid, Sigma, T5500
SDS	sodium dodecyl sulfate, Sigma, L3771
BHT	butylated hydroxytoluene, Sigma, B1378
Acetic acid	acetic acid, EMD, AX0073-59
t-buOOH	*tert*-butyl hydroperoxide, Sigma, 70% in water, B2633
Asc	l-ascorbic acid, Sigma BioXtra, ≥99.0%, crystalline, A5960
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Sigma, ≥99.5%, (titration), H3375
Vitamin K1	Sigma-Aldrich, BioXtra, ≥99.0% , (sum of isomers, HPLC), mixture of isomers
Methanol	Sigma-Aldrich, CHROMASOLV®, for HPLC, ≥99.9%
n-Hexane	EMD, HX0290-1 Hexanes, HPLC, 98.5%
Butanol	EMD, BX1780-5, 1-Butanol, 99.4%
Pyridine	EMD, B10225-74, Pyridine, ANALAR
Formic acid	EMD Chemicals, Formic Acid, ACS Grade, 98, Model FX0440-5

3.3. Liposome preparation

All the lipids for liposome (Lipo) preparation were mixed together in the absence of oxygen. To remove oxygen, solvents and solutions were saturated with N_2 by bubbling with pure N_2 for 10 min. To prepare liposomes first, 48 µl of the phosphatidylethanolamine solution (125 mg/ml, in N_2-saturated chloroform), 950 µl of cardiolipin (4.7 mg/ml, in N_2-saturated ethanol) and 75 µl of the phosphatidylcholine (100 mg/ml, in N_2-saturated ethanol) were added into a test tube. The original lipid container was re-flushed with N_2 after transferring lipids. For the vitamin E integrated liposomes (LipoE), α-tocopherol was diluted in N_2-saturated ethanol and added into the tube in different amounts. After the lipids, 3 ml of chloroform was added into the tube to dilute and distribute the lipids. After mixing the chloroform and lipids together, N_2 was blown into the angled and rotated tube to evaporate the solution and create a film. The tube was sealed with parafilm and it was frozen overnight. Before sonicating the lipids, 3.5 ml of N_2-saturated, cold 10 mM HEPES, 5 mM KPi buffer (pH 7.2) was added into the tube. The solution, on ice, was sonicated with the microtip probe at 50% amplitude (~100 Watts), for 8 X 15 second bursts with 45 seconds cool-down in between. This sonication changed the suspension from a cloudy opaque appearance to a more transparent appearance. The sonicated solution was centrifuged at 5000 g, 4 ^{o}C, for 20 minutes and any pelleted material (very little was visible) was discarded. Various concentrations of α-tocopherol were integrated into liposome (Table 4) in order to determine the optimal concentration to cooperate with acorbate and flavonoids, and also to compare with that of mitochondria.

Table 4. *Concentrations of α-tocopherol integrated into liposome*

Total α-tocopherol	Total lipid	α-tocopherol concentration
0.0252 mg	18 mg	0.0014 mg/mg
0.0504 mg	18 mg	0.0028 mg/mg
0.252 mg	18 mg	0.014 mg/mg
0.504 mg	18 mg	0.028 mg/mg
1.512 mg	18 mg	0.072 mg/mg

3.4. Mitochondria preparation

The Animal Care Protocol (#20030012) was approved by the University of Saskatchewan UCACS Protocol Review Committee. The mitochondria were prepared from the livers of adult male Wistar rats from the University of Saskatchewan Animal Resource Centre. All rats in the mitochondria study were from the same age group (body weight range: 250-350 g). The rats were decapitated after being anesthetized with isoflurane and livers were immediately excised and homogenized in mitochondrial preparation buffer 1 (250 mM sucrose, 10 mM Hepes, 1mM EGTA, pH 7.2). The mitochondria were isolated by differential centrifugation (117) and resuspended in buffer 3 (250 mM sucrose, 10 mM Hepes, pH 7.2). The mitochondria were frozen at -80^{o}C for later experiments on lipid peroxidation and vitamin E content.

3.5. Protein concentration measurement

Protein concentrations of the mitochondria preparations were measured by the Biuret method (118). The biuret reagent is made of 32 mM potassium sodium tartrate, 12 mM copper sulfate, 30 mM potassium iodide, and 0.2 M NaOH, whose combination will turn

violet with the presence of the protein. A 100 µl aliquot of freshly extracted, or thawed-resuspended mitochondrial samples were added into the 900 µl of the biuret reagent, vortexed and left for 20 minutes at room temperature and the absorbance read at 550 nm by spectrophotometer. Bovine serum albumin was used as a protein standard at 0, 10, 20 40 and 80 mg/ml with 5 replicates.

3.6. Ascorbate decay test

In order to determine whether ascorbate was present through the incubation of liposomes with t-buOOH, the loss of ascorbate was monitored spectrophotometrically at 265 nm in 50 mM KPi buffer (pH 7.2) at 37^{o}C. The conditions were the same as the control groups in the liposomes TBARS test (50 mM KPi buffer, pH 7.2 at 37^{o}C).

3.7. Thiobarbituric acid reactive substances assay (Liposomes)

For experiments on the effects of ascorbate and flavonoids on liposomal lipid peroxidation, 50 µl of lipo or lipoE was added into 200 µl of 50 mM KPi buffer, pH 7.2 with different treatments. All the samples were vortexed and incubated at 37 ℃ for the times indicated.

This method determines levels of reactive aldehydes such as malondialdehyde (MDA), a lipid oxidation product, by heating the samples in the presence of thiobarbituric acid (TBA) at low pH, resulting in the formation of a pink chromophore with an absorption maximum near 532 nm (119). We modified some details of the essay. From the incubations, 100 µl of the incubant was added to 400 µl of thiobarbituric acid solution containing 9.3% acetic acid (pH 3.5), 0.5% sodium dodecyl sulfate, 0.375% thiobarbituric acid, and 0.0002% butylated hydroxytoluene, and heated for 1 h at 95 ℃.

The heated samples were cooled and the TBA reaction products were extracted with an equal volume of the butanol/pyridine (15 : 1, v:v). The mixture was centrifuged at 4000 g for 10 minutes, and the fluorescence of the upper layer was measured at 530 nm (excitation of 485 nm). MDA stock solution (10 mM) was prepared by 96.5% sulfuric acid and further diluted with water to obtain final concentration of 0.1mM. A series of concentrations were used as standards for each batch of TBA.

The α-tocopherol content in mitochondria was fixed, while we could manipulate its concentration in liposomes. A series of concentrations of α-tocopherol were used to study its cooperation with ascorbate and flavonoids. The combinations tested were as follows (Table 5):

Table 5. *Tested combinations of antioxidants using liposomes*

α-tocopherol	Ascorbic acid	Flavonoids			
		Quercetin	Catechin	Rutin	Cyanidin
0.0014 mg/mg or 0.0028 mg/mg or 0.014 mg/mg or 0.028 mg/mg or 0.072 mg/mg	200 μM or 400 μM	100 μM or 50 μM	100 μM or 50 μM	50 μM	50 μM

Note: The combination of α-tocopherol (5 concentrations), ascorbic acid (2 concentrations) and each type of flavonoids (2 or 1 concentration) was studied, but not the combination of different types of flavonoid. Each combination contained at least one experiment (with at least 3 replicates), and some results were not shown in the thesis.

3.8. Thiobarbituric acid reactive substances assay (Mitochondria)

The basic experimental setup was the same as the TBARS test for liposomes, except as specified below. Thawed mitochondria suspensions were centrifuged at 12,000 g, 4^{o}C, to remove the buffer containing sucrose, as it interferes with the TBARS results, and the pellet was resuspended in the same volume of 1.15% KCl. This suspension was added into 50mM KPi buffer (pH 7.2) to reach the final protein concentration together with different treatments.

In order to compare with the liposome studies, the combinations in Table 5 were also repeated in mitochondria, but only with fixed α-tocopherol content (self-integrated in mitochondria). In addition, 100 μM of rutin and cyanidin were also tested in combination with 200 μM & 400 μM ascorbic acid.

Since α-tocopherol was integrated in mitochondria, we applied the combination of acorbate and flavonoids in this system to investigate their influence on α-tocopherol content. All the samples were vortexed and incubated at 37℃ for exactly 60 minutes. Aliquots of 100 μl were taken for measurements of TBARS, and in the experiments involving LC/MS analysis of α-tocopherol, the rest of the incubant (9.9 ml from 10 ml) was collected to extract α-tocopherol in the mitochondrial membranes (extraction method in the next section).

3.9. HPLC-MS/MS analysis in measuring mitochondrial α-tocopherol

The incubated samples were centrifuged at 12,000 g to collect the mitochondria. After centrifugation, the supernatant was carefully removed by Pasteur pipette and 20 μl of 300 pM internal standard (vitamin K_1) was added into the samples. In order to extract both α-

tocopherol and internal standard, 1 ml of *n*-hexane:methanol (5:2) was added into the centrifuge tube and the pellet attached to the wall was washed off by pipettor. The samples were further vortexed for 1 minute, centrifuged at 150g for 1min and the upper *n*-hexane layer was carefully transferred to glass tubes. The extraction was repeated another two times. All the *n*-hexane samples collected were put into a freeze dryer overnight and the extract powder was redissolved in 200 µl of mobile phase and filtered with a 0.2 µm PTFE filter before LC/MS analysis. Under these conditions, the analytes were stable with over 85% recoveries.

HPLC grade α-tocopherol and vitamin K_1 were purchased from Sigma-Aldrich and dissolved in mobile phase separately, to 375 nM and 125 nM, as stock solutions. Four concentrations of α-tocopherol were further diluted to standard samples as listed in Table 6, and analyzed by HPLC-tandem mass spectrometry. The Lower Limit of Quantification (LLOQ) of α-tocopherol was determined as 20 pM, at which concentration yielded peak heights 10 times higher than the noise baseline.

Table 6. *Analyte concentrations in the standard curve*

	α-tocopherol	Vitamin K_1
Blank	0	0
Double Blank	0	30 pM
Standard 1	20 pM	30 pM
Standard 2	100 pM	30 pM
Standard 3	500 pM	30 pM
Standard 4	1 nM	30 pM

Tandem mass spectrometry with an HPLC system was used to quantify the α-tocopherol in rat liver mitochondria. HPLC separation of α-tocopherol was achieved on a Varian Pursuit column (Varian Pursuit XRS, 5 micron C18, 250 mm x 3.0 mm column) using an Agilent 1100 HPLC system. Mobile phase A was Milli-Q water with 0.1% formic acid, mobile phase B was methanol with 0.1% formic acid. They were filtered through a 0.45 μm membrane filter prior to analysis. The mobile phase was pumped at 0.8 mL/min for 30 minutes per injection with an isocratic proportion of 0.5% A and 99.5% B. The column temperature was held constant at 30 ℃ and the sample injection volume was 10 μL for all injections.

MS/MS analysis was performed on a Qtrap 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA) equipped with a heated nebulizer inlet (APCI). The APCI source operated in positive ion mode, and the following settings were used: corona discharge current, 4.0 μA; source temperature, 400 ℃; collision-activated collision gas (nitrogen) setting, 6 (arbitrary units); curtain gas (nitrogen) setting, 6; and nebulizer gas (nitrogen) setting, 10. MRM measurements with the transitions APCI-MS (+) m/*z* (431.3 $\rightarrow$165.1) for α-tocopherol and 451.3 $\rightarrow$187.3 for vitamin K_1 were determined using declustering potential, entrance potential, collision energy, and collision cell exit potential values at 66 V, 10 V, 27 eV, 12 V and 71 V, 10 V, 31 eV, 14 V respectively. The parent-daughter ion pairs are summarized in the table & figure below.

Table 7. *List of molecular weight (MW), observed molecular ion (Q1), fragment ion (Q3), and characteristic neutral loss of different analytes under positive APCI condition*

Analyte	MW[Da]	Q1 m/z	Q3 m/z	Characteristic mass loss [Da]
α-tocopherol	430	431	165	266
Vitamin K_1	450.7	451.5	187.1	264

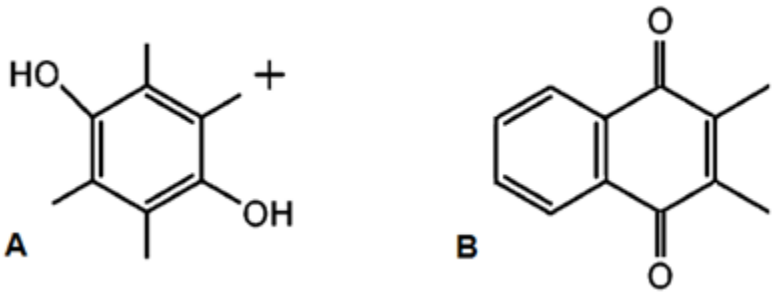


Figure 9. *The daughter ions of α-tocopherol (A) and vitamin K_1 (B). Mass over charge ratio for A, 165; B, 187.3.*

Before MS was applied, we tried using HPLC with fluorescence detection to determine the α-tocopherol content in the mitochondria. However, due to low sensitivity and specificity, the method was abandoned. Also, we tried to measure the γ-tocopherol content in the mitochondria with the tandem MS, as γ-tocopherol is the most abundant source of vitamin E in the North American diet (48, 120), and it also exists in the mitochondrial lipid membrane system (121). The combined concentration of α- and γ-tocopherol would generate a bigger picture of the vitamin E status in the mitochondria membrane system. Although we optimized the parameters for its analysis and established the methods with a standard curve and LLOQ, the amount of γ-tocopherol was lower than the lowest quantitative amount. These results are shown in Appendix 1.

3.10. Statistical Analysis

Descriptive statistical analyses were performed using Excel software (Microsoft Office 2007) for the means and the standard error of the mean (SEM). Results were expressed as the mean ± SEM (or mean ± SD when only one liposome preparation was tested). The significance level was set at *p* less than 0.05. Using SPSS software for Windows (version 19) the data were analyzed by one-way analysis of variance (ANOVA). Differences among treatment and control groups were determined by the multiple range *post hoc* test, Tukey's Honestly Significant Differences (HSD). Interactive effect was determined by two-way ANOVA. When the *p* value of factor A*B was less than 0.05, an interaction of factor A and B was reported. Pearson correlation coefficient was computed by SPSS to assess the relationship between the amount of α-tocopherol integrated in liposomes and TBARS formation. Pearson's correlation (r) and corresponding *p* value were reported as well.

4. RESULTS

4.1. Pro-oxidant effect of ascorbate

4.1.1. Pro-oxidant effect of ascorbate on liposomes

In order to confirm the pro-oxidant effect of ascorbate in the liposome model, different levels of ascorbate were added alone to vitamin E integrated liposome. With increasing concentration of ascorbate added into the incubation, the TBARS generation increased accordingly. Ascorbate at 400 μM was able to elevate the liposomal oxidative status to a significant level, while 200 μM of ascorbate did not make a statistical difference compared to the control group (Figure 10).

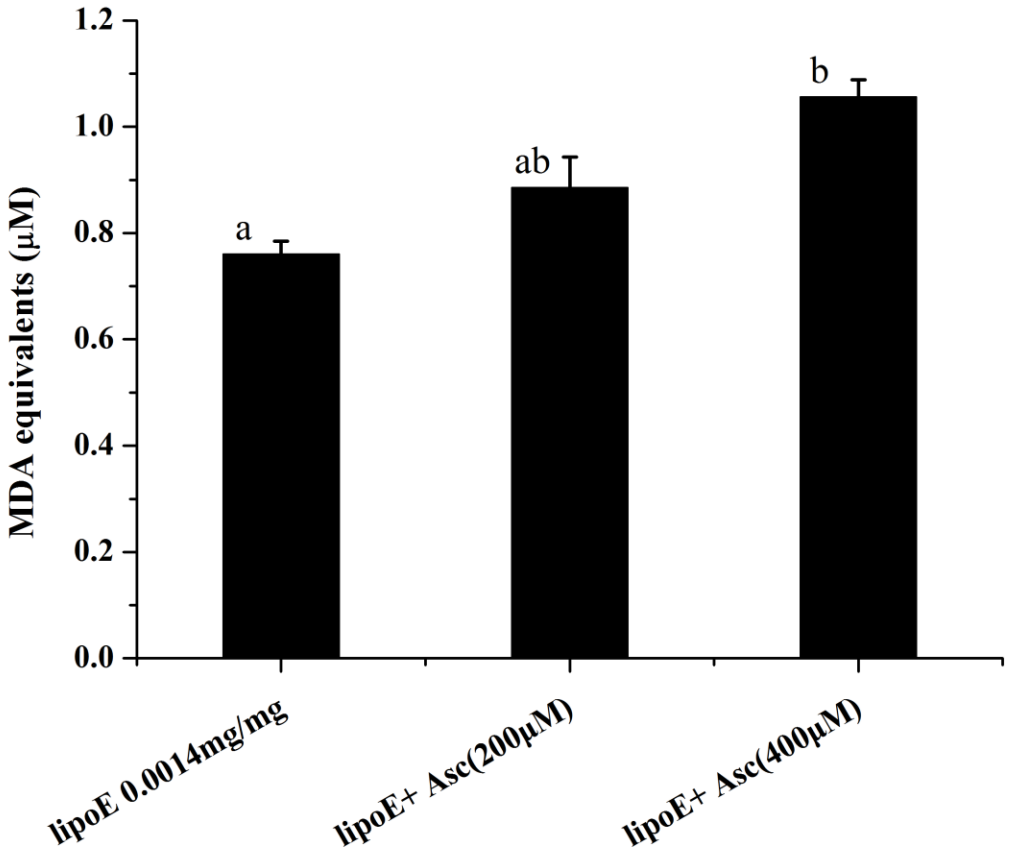


Figure 10. *TBARS generation in α-tocopherol integrated liposomes treated with different amounts of ascorbic acid*. Liposomes were incubated in 50 mM potassium phosphate buffer (pH 7.2) at 37^{o}C for 1 hour under different conditions (α-tocopherol 0.014 mg/mg in total lipids), asc – ascorbic acid 200 µM/ 400 µM, n=3 preparations). ^{ab} Different letters show significant differences by one-way ANOVA (post-hoc by Tukey's HSD) at $p<0.05$.

4.1.2 Pro-oxidant effect of ascorbate on rat liver mitochondria

To investigate whether ascorbate was able to induce a pro-oxidant effect on mitochondria *in vitro*, different concentrations of ascorbate were incubated with mitochondria. The TBARS formation significantly increased with the presence of the ascorbate and this trend was enhanced by its higher concentration (Figure 11).

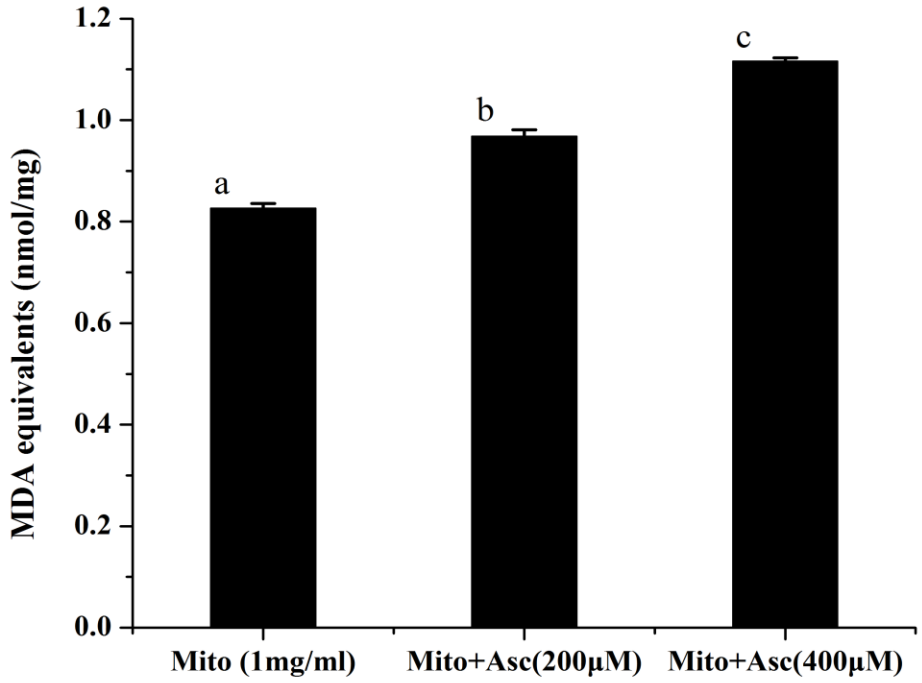


Figure 11. *Pro-oxidant effect of ascorbic acid on mitochondria.* Liposomes were incubated in 50 mM potassium phosphate buffer (pH 7.2) at 37^{o}C for 1 hour under different conditions (α-tocopherol 0.014 mg/mg in total lipids), Asc – ascorbic acid 200 µM/ 400 µM, n=3 preparations). ^{abc} Different letters show significant differences by one-way ANOVA (post-hoc by Tukey's HSD) at $p<0.05$.

4.1.3 Limitation of pro-oxidant effect of ascorbate in liposomes by quercetin

The ability to reduce the pro-oxidant effect of ascorbate by quercetin was tested by the TBARS test in liposomes without vitamin E (Figure 12). Adding 50 µM quercetin alone had no significant effect on the liposome oxidation status. In the presence of ascorbate, further addition of quercetin was able to bring down the TBARS formation significantly, indicating that quercetin was efficient in suppressing the pro-oxidant effect of ascorbate.

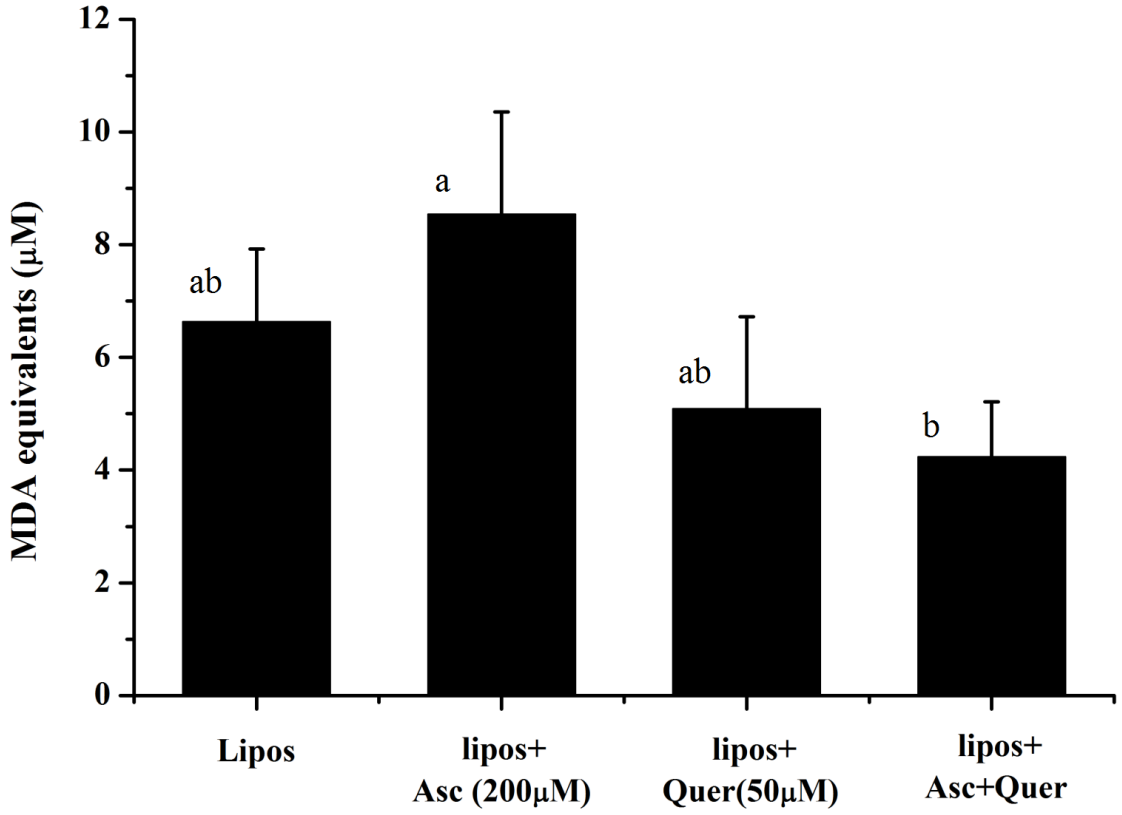


Figure 12. *TBARS generation in liposomes under treatment of ascorbic acid and quercetin*. Liposomes were incubated in 50 mM potassium phosphate buffer (pH 7.2) at 37^{o}C for 1 hour under different conditions, Asc – ascorbic acid 200 μM, Quer – quercetin 50 μM, n=3 preparations). [ab] Different letters show significant differences by one-way ANOVA (post-hoc by Tukey's HSD) at $p<0.05$).

4.2. Effects of different flavonoids, ascorbate and vitamin E in t-buOOH-induced TBARS

4.2.1. Ascorbate content during incubation

The ascorbate decay test was performed to determine how long ascorbate was present during the incubation and the minimum initial concentration to ensure its presence during the one-hour incubation for TBARS experiments. After adding ascorbate (final concentration 200 μM) into the suspension of liposomes without α-tocopherol (Figure

13A), the absorbance went up to 2.5 and upon addition of peroxide, the value dropped quickly. At 27 minutes, the reading was stable at about 1.5. To verify whether ascorbate was all consumed, 10 μM Cu^{2+} were added due to its ability to catalyze ascorbate oxidation by oxygen, and the value did not drop any further. The remaining absorbance was presumably due to lipids and peroxide-induced formation of conjugated dienes (with λmax = 235 nm) (122). We could conclude that the ascorbate is all oxidized after 20 minutes of incubation with t-buOOH under these conditions. In Figure 13B, when adding ascorbate into the suspension of liposomes with α-tocopherol, the presence of ascorbate was extended and was not significantly consumed after 30 minutes incubation with t-buOOH until adding Cu^{2+} (Figure 13B).

As 200 μM of ascorbate was consumed in less than one hour incubation, I doubled the concentration and measured again. Figure 14A and B showed that in both types of liposomes, the 400 μM of ascorbate was able to last through one hour of incubation. Therefore, this concentration was chosen for the later TBARS analysis.

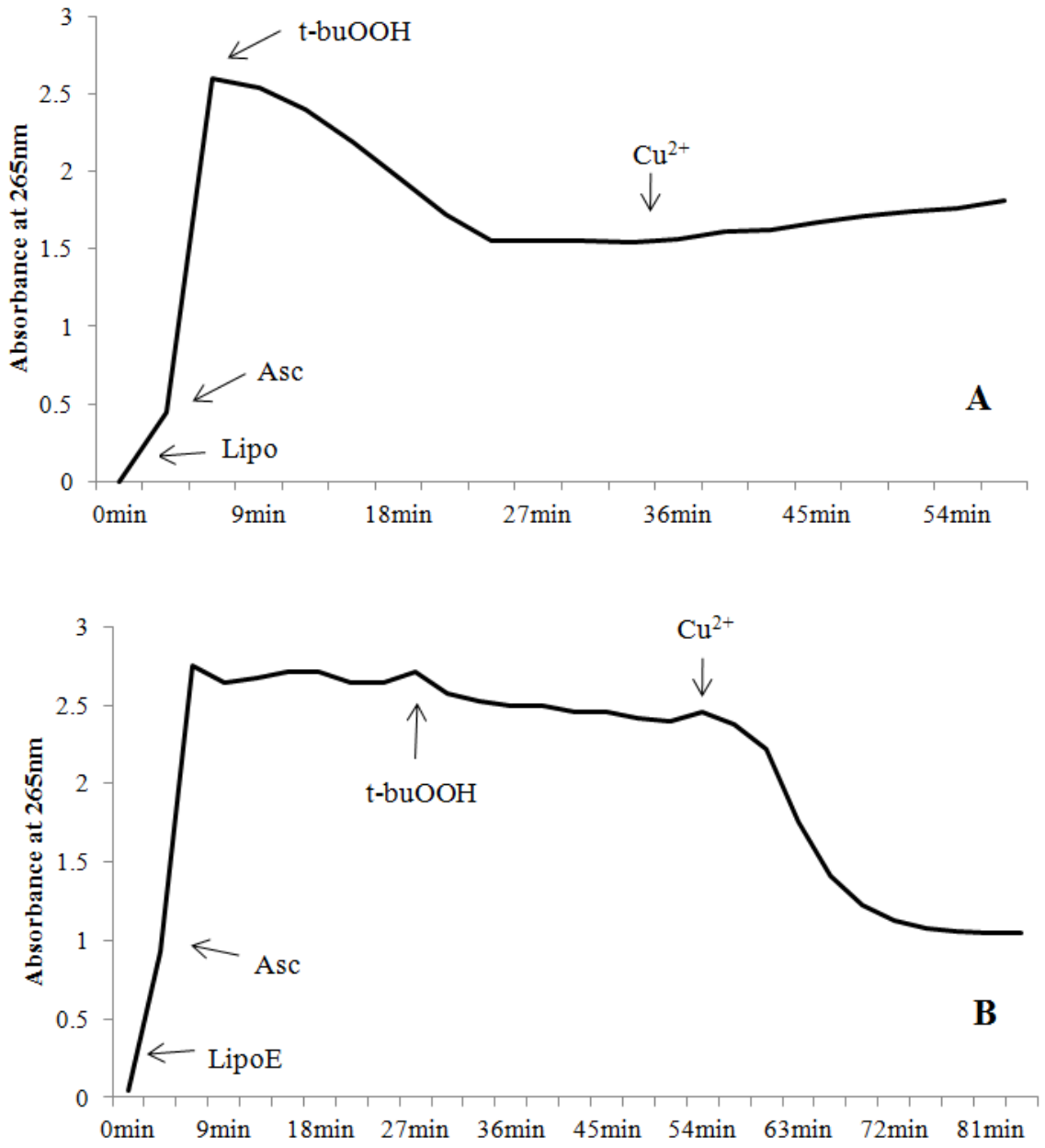


Figure 13. *Peroxide-induced loss of 200 μM ascorbic acid in the presence of liposomes.* Ascorbic acid (Asc, 200 μM, t-buOOH, 1 mM, Cu^{2+}, 10 μM). **A**. Liposomes without α-tocopherol. **B**. Liposomes with 0.072 mg/mg of α-tocopherol in total lipids. Loss of ascorbate was monitored spectrophotometrically at 265 nm in 50 mM potassium phosphate buffer (pH 7.2) at 37°C with additions of liposomes (18 mg/ml) total lipids), ascorbic acid (Asc, 200 μM), *tert*-butyl hydroperoxide (t-buOOH – 1 mM) and $CuSO_4$ (Cu^{2+}, 10 μM) as indicated.

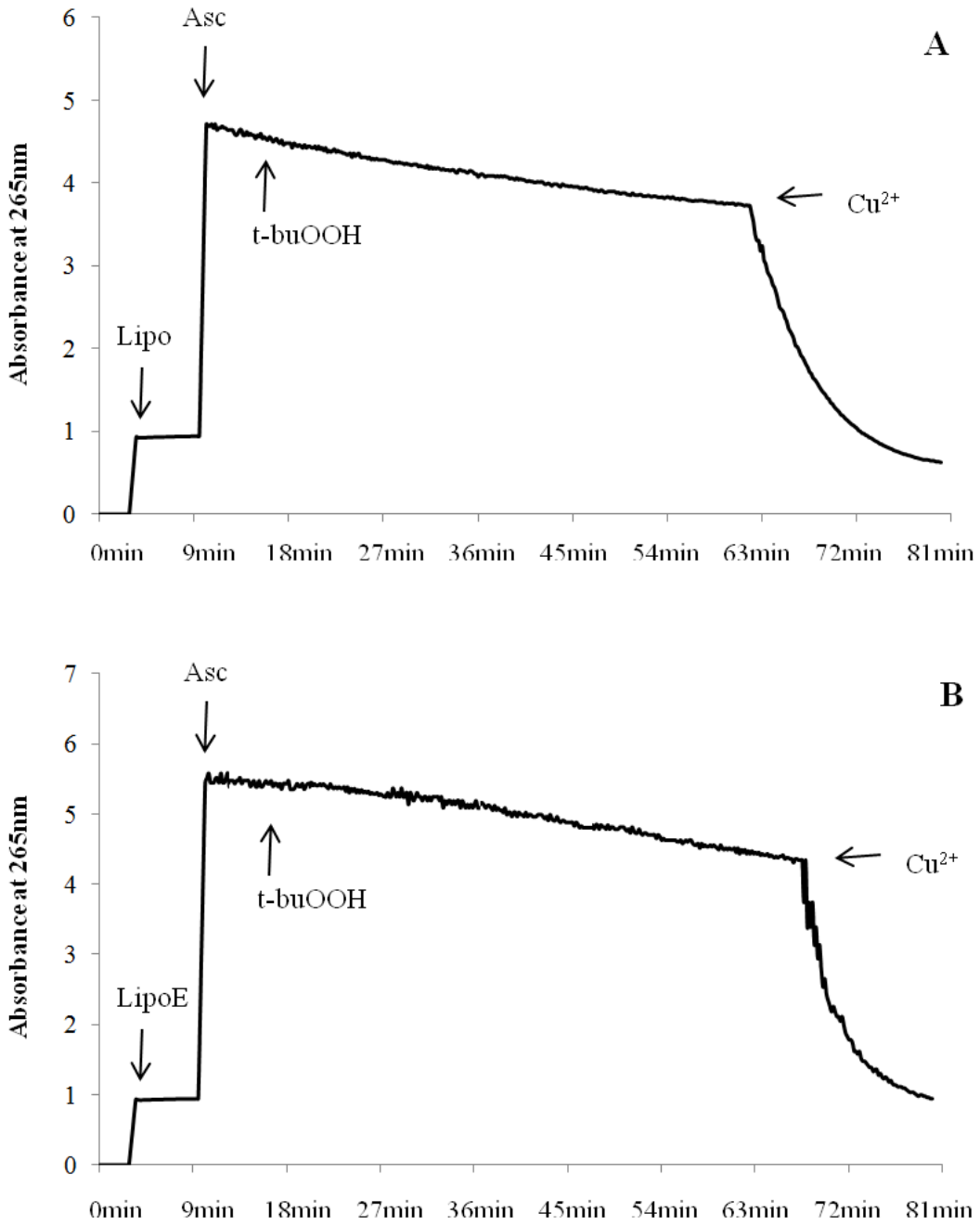


Figure 14. *Peroxide-induced loss of 400 μM ascorbic acid in the presence of liposomes*, **A**. Liposomes without α-tocopherol. **B**. Liposomes with 0.072 mg/mg of α-tocopherol in total lipids. Measurements were conducted in the same condition as in the previous figure. Loss of ascorbic acid was monitored spectrophotometrically at 265 nm in 50 mM potassium phosphate buffer (pH 7.2) at 37°C with additions of liposomes (18 mg/ml) total lipids), ascorbic acid (Asc, 400 μM), tert-butyl hydroperoxide (t-buOOH – 1 mM) and $CuSO_4$ (Cu^{2+}, 10 μM) as indicated. In these cases the measurements were conducted with 0.5 cm path length cuvettes and the absorbance values were doubled to give the theoretical absorbance at 1 cm path length.

4.2.2 Effects of different flavonoids, ascorbate and vitamin E in liposomal TBARS

In addition to quercetin, I explored (liposome with α-tocopherol concentration 0.072 mg/mg lipids) other flavonoids from several classes: rutin (quercetin glycoside), catechin (flavonol) and cyanidin (anthocyanidin). The following results compared the relative abilities of different flavonoids to cooperate with ascorbate and vitamin E in preventing TBARS formation. Without the presence of the α-tocopherol, the flavonoids alone could not establish any protection (Figure 15). In the presence of ascorbate, only quercetin was able to bring down the TBARS generation to a significant level with the *p* value of 0.015 (one-way ANOVA) comparing to the DMSO group (used in the quercetin stock solution). Notably however, t-buOOH did not increase the TBARS significantly, as the control liposomes already had a high level of TBARS in this preparation. Also, ascorbate did not increase t-buOOH-induced TBARS in this experiment with vitamin E-absent liposomes as was observed with some preparations (results not shown).

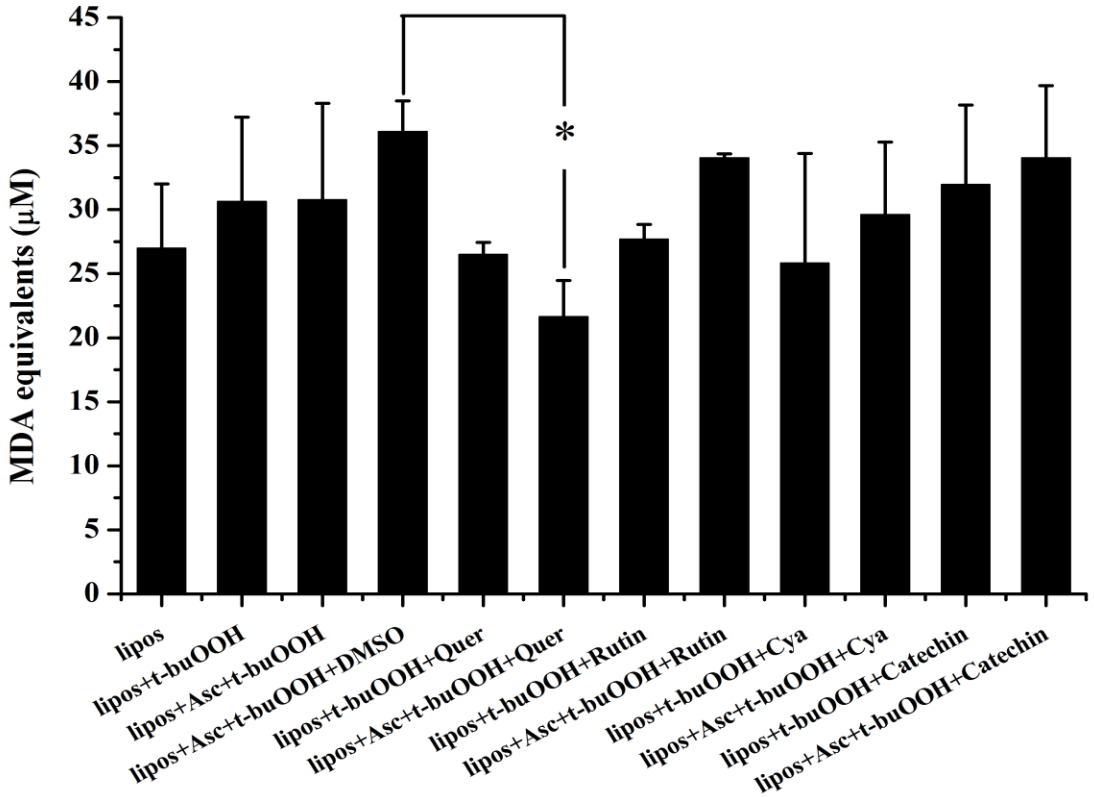


Figure 15. *Effects of different flavonoids and ascorbate on TBARS generation in liposomes without α-tocopherol.* Liposomes were incubated in 50 mM potassium phosphate buffer (pH 7.2) at 37^{o}C for 1 hour under different conditions, (Quer- quercetin; Rutin; Cya – cyanidin; Catechin 50 μM) Bars represent means ± SD of n=3 replicate incubations from one liposome preparation. * Star shows significant differences tested by one-way ANOVA (post-hoc by Tukey's HSD) at $p<0.05$.

With the presence of vitamin E (0.072 mg/mg lipids), the baseline TBARS was significantly lower than observed in liposomes without vitamin E (Figure 16 versus Figure 15), indicating that vitamin E was preventing lipid auto-oxidation during the liposome preparation. In incubations of these vitamin E-containing liposomes with t-buOOH, flavonoids were not able to protect the liposomes either with the presence or the absence of ascorbate (Figure 16).

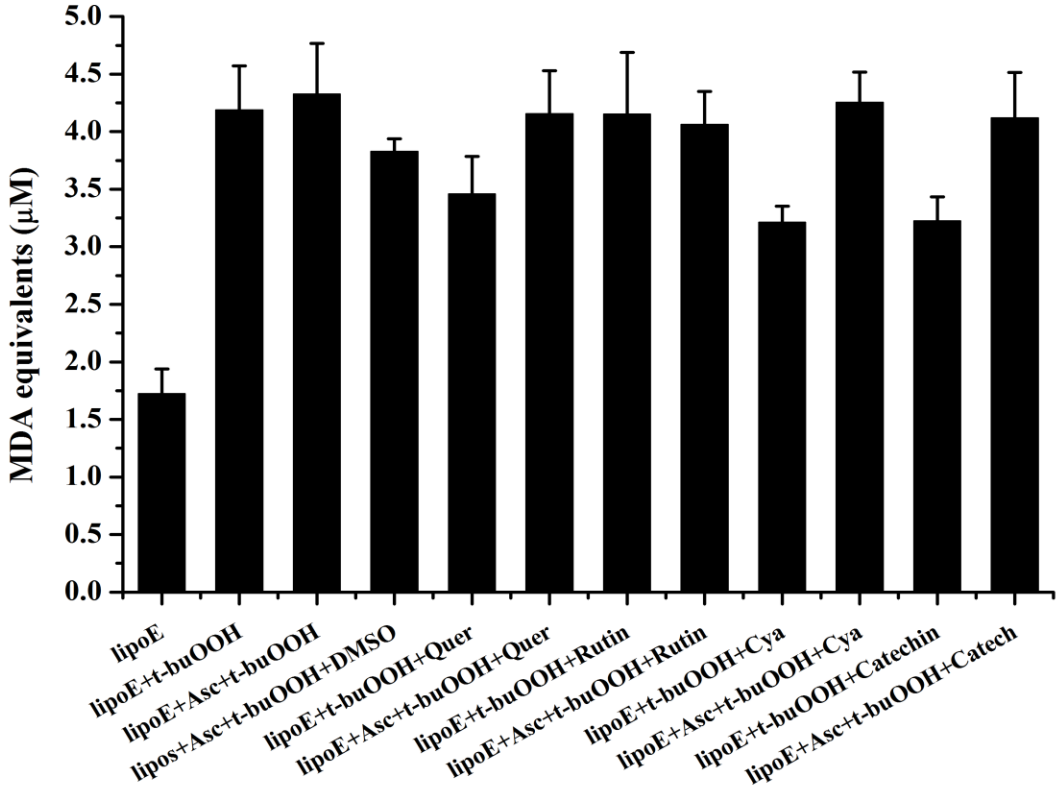


Figure 16. *Effects of different flavonoids and ascorbic acid on TBARS generation in liposomes with 0.072 mg/mg α-tocopherol*. Alpha-tocopherol-containing liposomes were incubated in 50 mM potassium phosphate buffer (pH 7.2) at 37^{o}C for 1 hour under different conditions, (Quer- quercetin, Rutin, Cya – cyanidin and Catechin 50 μM; n=3 preparations).

Utilization of the liposome system allows us to integrate different levels of vitamin E into the lipid membrane. Hypothetically, the antioxidant property of flavonoids may perform better when vitamin E is insufficient. If the amount of vitamin E could not prevent the oxidation of relatively larger amount of lipids, then cooperation with the hydrophilic antioxidants can be helpful in regenerating vitamin E and sustain the protection within the lipid membrane. However, in experiments with α-tocopherol at 0.0028 mg/mg lipid, the expected synergism was again not observed (Figure 17). Due to the apparent pro-oxidant effect of vitamin E (discussed later), the baseline TBARS level (liposomes

without t-buOOH) was lower compared to the results of higher vitamin E content liposomes (Figure 17 versus Figure 16). Similar to the results from high vitamin E liposomes, adding ascorbate into the samples during incubation with peroxide does not affect the lipid peroxidation. With peroxide, the liposomes were consistently oxidized to a low level (~2 μM MDA equivalents) through all the treatment groups, regardless of presence of ascorbate or flavonoids.

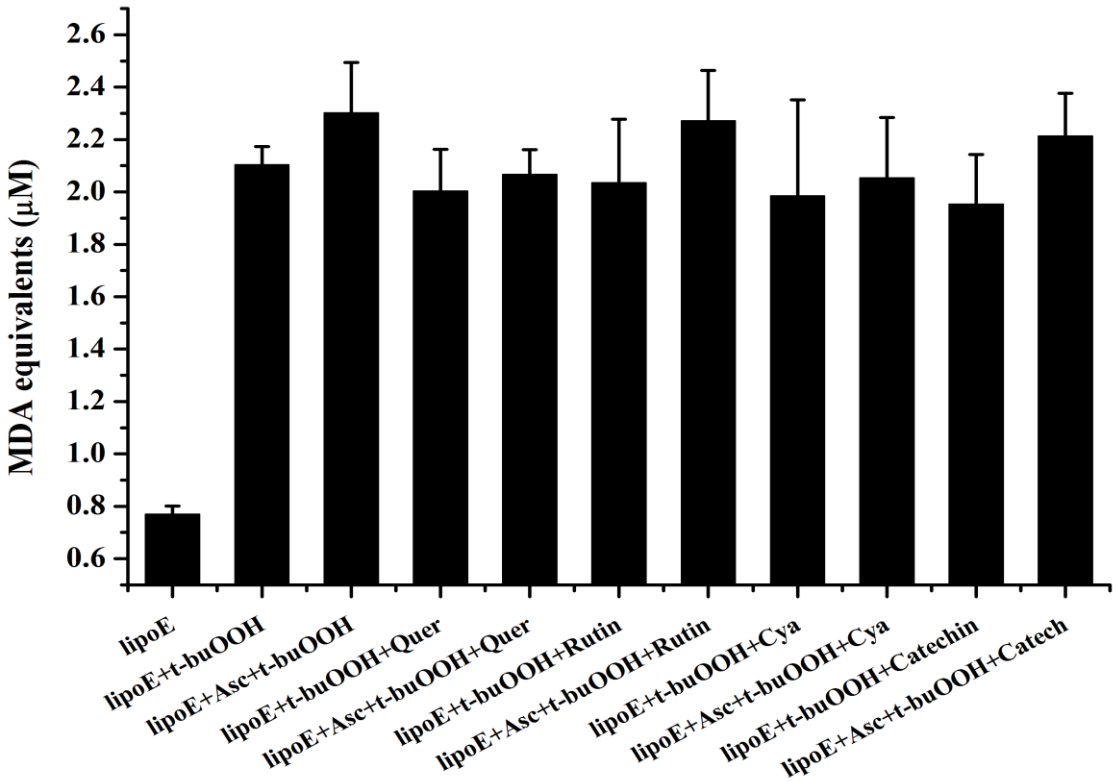


Figure 17. *Effects of different flavonoids and ascorbic acid on TBARS generation in liposomes with 0.0028 mg α-tocopherol/mg lipid.* Alpha-tocopherol-containing liposomes were incubated in 50 mM potassium phosphate buffer (pH 7.2) at 37^{o}C for 1 hour under different conditions, (Quer- quercetin, Rutin, Cya – cyanidin and Catechin 50 μM; n=4 repeats in one preparation of liposomes).

We also tested for the synergism in the presence of cytochrome *c* to determine whether its presence would induce synergism by enhancing the peroxidation process (Figure 18). The cytochrome *c* accelerated oxidation but did not induce synergism.

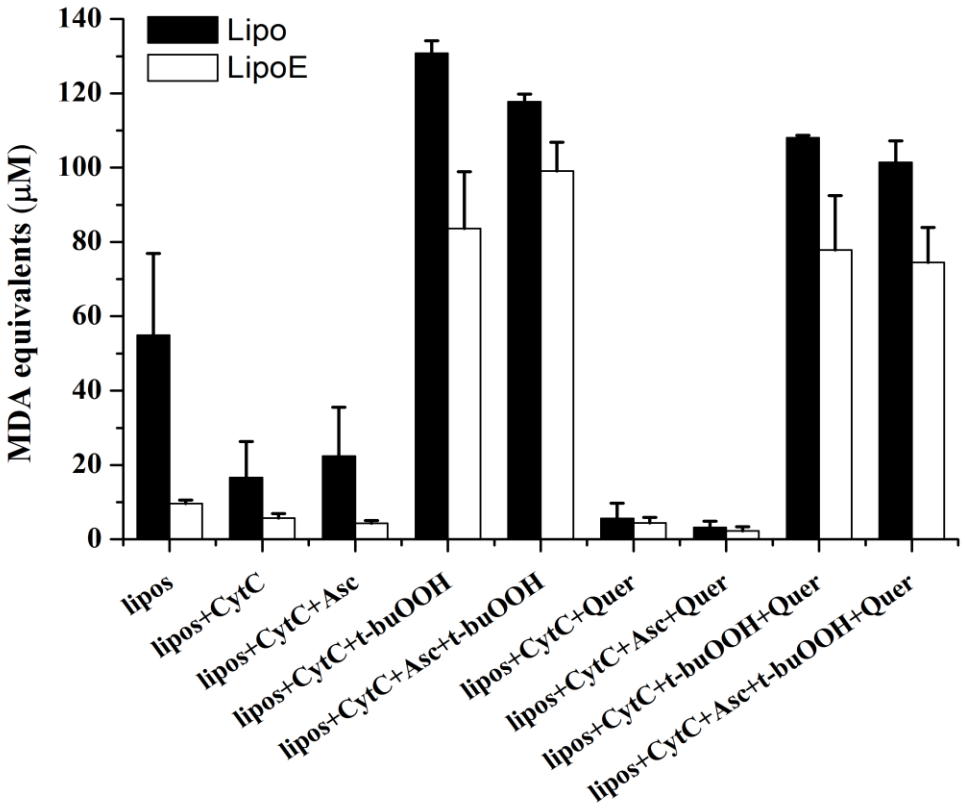


Figure 18. *Effects of ascorbic acid, quercetin and vitamin E on cytochrome c-catalyzed TBARS generation.* Liposomes were incubated for 1 hour in 50 mM potassium phosphate buffer (pH 7.2) at 37°C with the presence of 20 μM cytochrome c in different conditions (α-tocopherol 0.028 mg/mg in total lipids), CytC – cytochrome c 20 μM, Asc – ascorbic acid 200 μM, n=3 repeats in one preparation of liposomes).

According to previous results showing synergism with ascorbate in transmitting protection to a membrane environment (94), quercetin was a good candidate in synergistically regenerating vitamin E with ascorbate. Therefore, a large amount of time has been spent on searching the optimal conditions. In Table 8, all tested combinations of treatments are summarized and there was no synergistic protection on the liposomes (data

not shown in the thesis) except an additive protection by ascorbate (400 µM) and quercetin (100 µM) at the lowest α-tocopherol level (0.0014 mg/mg lipid) (Figure 19).

Table 8. *List of combination of different concentrations of α-tocopherol, ascorbic acid and quercetin in TBARS tests of liposomes undergoing t-buOOH-induced lipid peroxidation.*

α-tocopherol/total Lipids	Asc	Quer
0.0014 mg/mg	200 µM	100 uM
0.0014 mg/mg	400 µM	100 uM
0.0028 mg/mg	200 µM	50 uM
0.0028 mg/mg	400 µM	50 uM
0.0028 mg/mg	200 µM	100 µM
0.0028 mg/mg	400 µM	100 µM
0.014 mg/mg	200 µM	50 µM
0.014 mg/mg	400 µM	50 µM
0.014 mg/mg	200 µM	100 µM
0.014 mg/mg	400 µM	100 µM
0.028 mg/mg	200 µM	50 µM
0.072 mg/mg	200 µM	50 µM
0.072 mg/mg	400 µM	50 µM

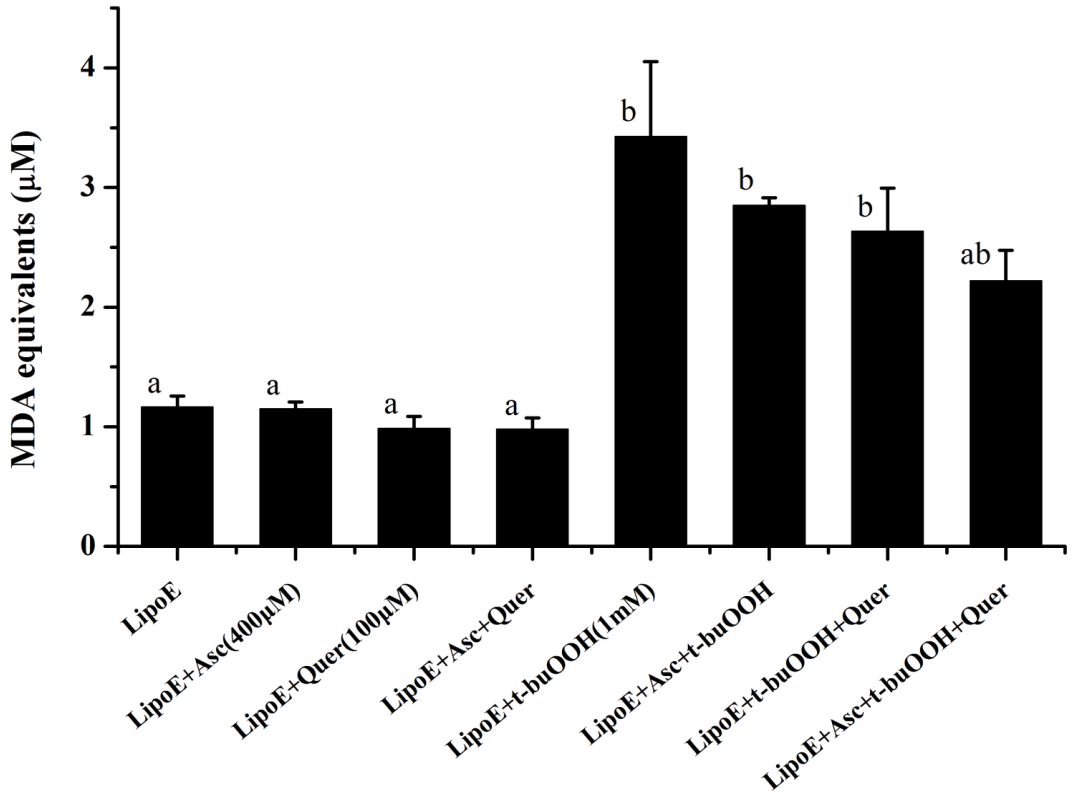


Figure 19. *Cooperative protection from ascorbic acid and quercetin on α-tocopherol integrated liposomes.* Liposomes were incubated with KPi buffer (pH 7.2, 50 mM) for one hour (α-tocopherol 0.0014 mg/mg in total lipids, Asc – ascorbic acid 400 μM, Quer – quercetin 100 μM, n=3 preparations with double measurements for each preparation). Bars represent mean ± SEM. [ab] Different letters show significant differences by one-way ANOVA (post-hoc by Tukey's HSD) at $p<0.05$.

The TBARS formation in the group protected by both ascorbate and quercetin was lower than the groups treated with either of them, and also lower than the group treated with peroxide alone. Although there was no significant decrease in oxidation level by this antioxidant team, their combination was still able to bring down the oxidative stress level to which there was no longer significant difference from the baseline groups.

Although we tried different methods to reduce the baseline TBARS in the liposomes after sonication, including flushing nitrogen into the buffer before and after preparation, keeping everything on ice through the process, separating the sonication with a cool-

down period and centrifuging out the oversized liposomes after sonication, the liposomes without any treatment still had inconsistent and often elevated TBARS. In most preparations, the application of peroxide did not bring up the oxidation significantly, indicating that although the liposome was protected by all means through the preparation, it was still auto-oxidized during the sonication or by the air incubation. On the other hand, vitamin E provided sufficient protection to the liposomes through the sonication and air incubation. Comparing the scale of the TBARS results in Figure 15 and 16, we can see that without vitamin E, all the treatment groups had TBARS formation to around 20 to 40 μM while the treatment groups within the vitamin E-containing liposomes were constrained within 1.5 to 4 μM of MDA equivalency.

4.2.3. Pro-oxidant effect of α-tocopherol

The previous research (Figures 14 and 16) was using 0.13 mg α-tocopherol per 18 mg lipid (0.072 mg/mg). Over the course of the research I tried different amounts of α-tocopherol incorporated into liposomes, and interestingly the vitamin E protection from air induced oxidation was not proportional to its concentration. There was a statistically significant positive relationship between the TBARS generation during incubation and the amount of α-tocopherol (although statistical differences among the vitamin E groups were not evident using one-way ANOVA) (Figure 20).

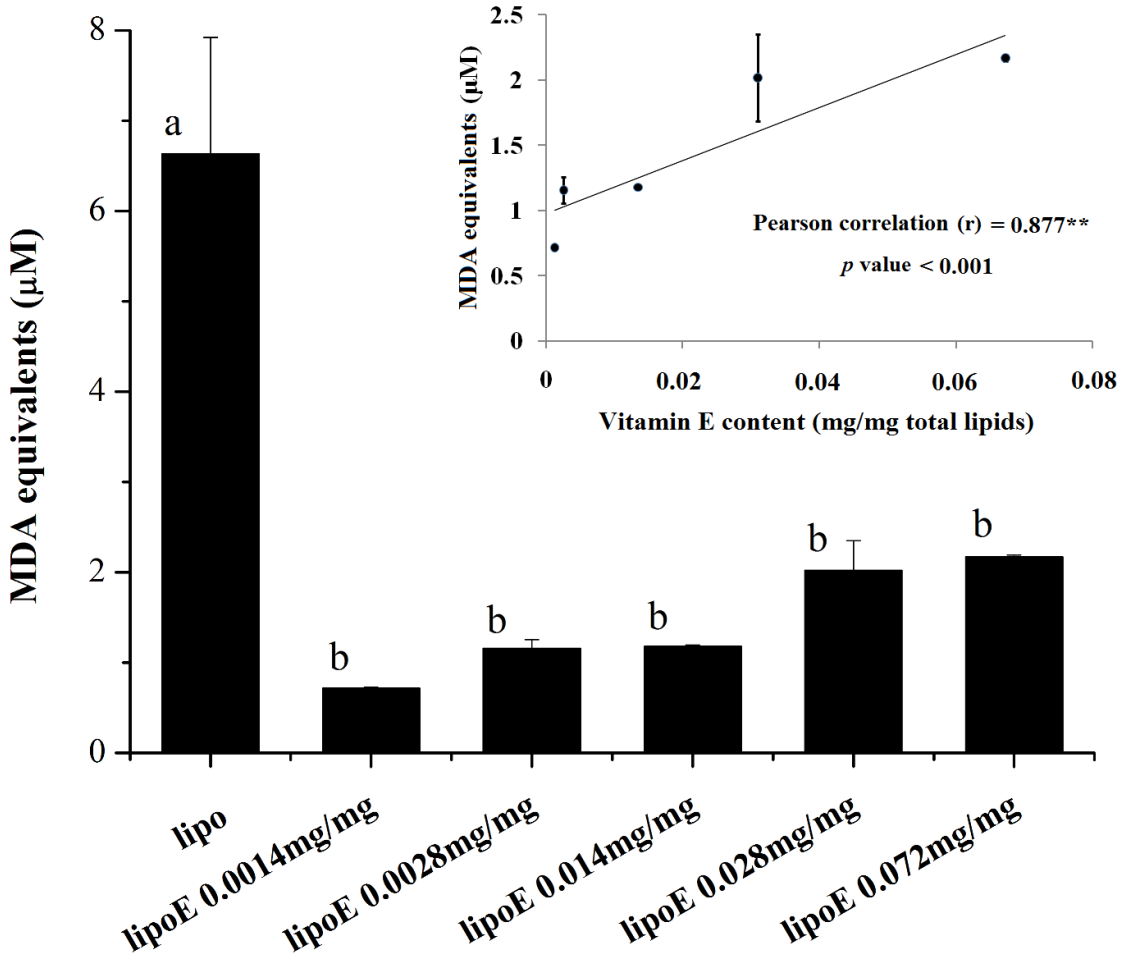


Figure 20. *TBARS generation in liposomes integrated with different amounts of α-tocopherol.* Liposomes were incubated with KPi buffer (pH 7.2, 50 mM) for one hour. Bars represent means ± SEM (n=3 preparations). [ab] Different letters show significant differences by one-way ANOVA (post-hoc by Tukey's HSD) at $p<0.05$). The inset shows the correlation between TBARS and increasing vitamin E content. **Statistically significant correlation at $p < 0.01$.

4.2.4 Effects of flavonoids and ascorbate on mitochondrial TBARS

As the membrane contents in the two systems were measured differently (measured by lipid in liposomes and by protein in mitochondria), it was necessary to unify the two units in order to compare the amount of lipids that were subject to oxidation. According to Alberts et al. (123), the estimated overall weight ratio of protein-to-phospholipid in the mitochondrial membranes is around 2:1. With the liposomes, the final phospholipids concentration in the incubation was 1mg/ml. According to the ratio, 2 mg/ml protein of mitochondria suspension was equivalent to the liposome incubations as of lipid content.

The original peroxide concentration (1 mM) was not able to induce significant oxidation in mitochondria in one hour (results not shown), perhaps due to the presence of peroxidases in mitochondria; therefore a higher concentration (5 mM) was used. The results from the TBARS test showed that the flavonoids alone gave significant protection against t-buOOH-induced TBARS (inhibiting by 27-40%), but there were still no synergistic effects by any flavonoids and ascorbate combination in protecting the mitochondria (Figure 21). The pattern that further addition of ascorbate in the presence of flavonoids exacerbated the oxidation was similar in both liposome (Figure 16 & 17) and mitochondria systems (Figure 21). The group treated with peroxide and ascorbate was considered to be the positive control and due to the small standard error in each group, it was significantly different from all the flavonoids treated groups except the group of rutin and ascorbate combination (with a trend of p=0.063).

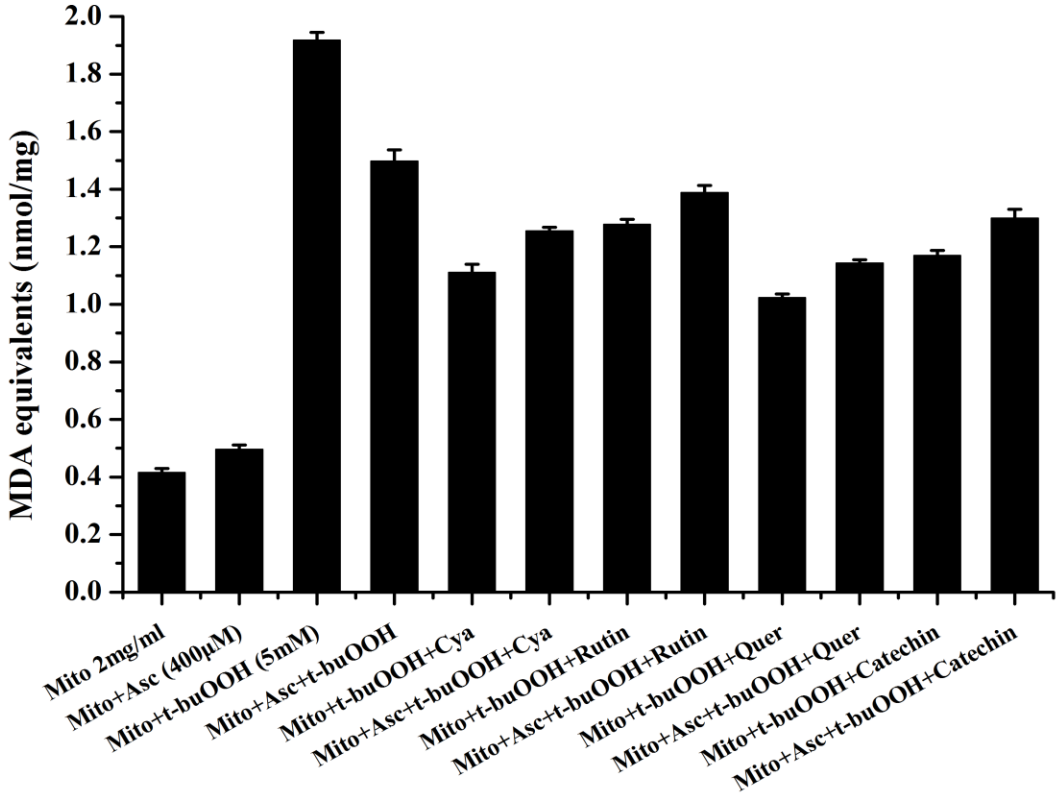


Figure 21. *Effects of different flavonoids and ascorbic acid on TBARS generation in mitochondria.* Mitochondria (2 mg/ml) were incubated with KPi buffer (pH 7.2, 50 mM) for one hour (t-buOOH 5 mM, Asc – ascorbic acid 400 μM, Quer – quercetin, Catechin, Rutin and Cya - cyanidin 50 μM, n=4 preparations with at least double measurements for each preparation). Bars represent means ± SEM. Analyzed by one-way ANOVA (post-hoc by Tukey's HSD) at $p<0.05$. All antioxidant treated groups (Group 4 - 12) had significant lower TBARS generation compared to the peroxide alone treated group (Group 3), while the TBARS values in the flavonoid + ascorbic acid combination groups were not different from groups treated with either antioxidants.

Quercetin and catechin were also tested in experiments with mitochondria at 1 mg protein/ml (Figure 22). However the difference in the amount of incubated mitochondria did not affect the TBARS results. The quercetin or ascorbate alone inhibited significantly (47% and 37%), but their combined presence did not improve the inhibition.

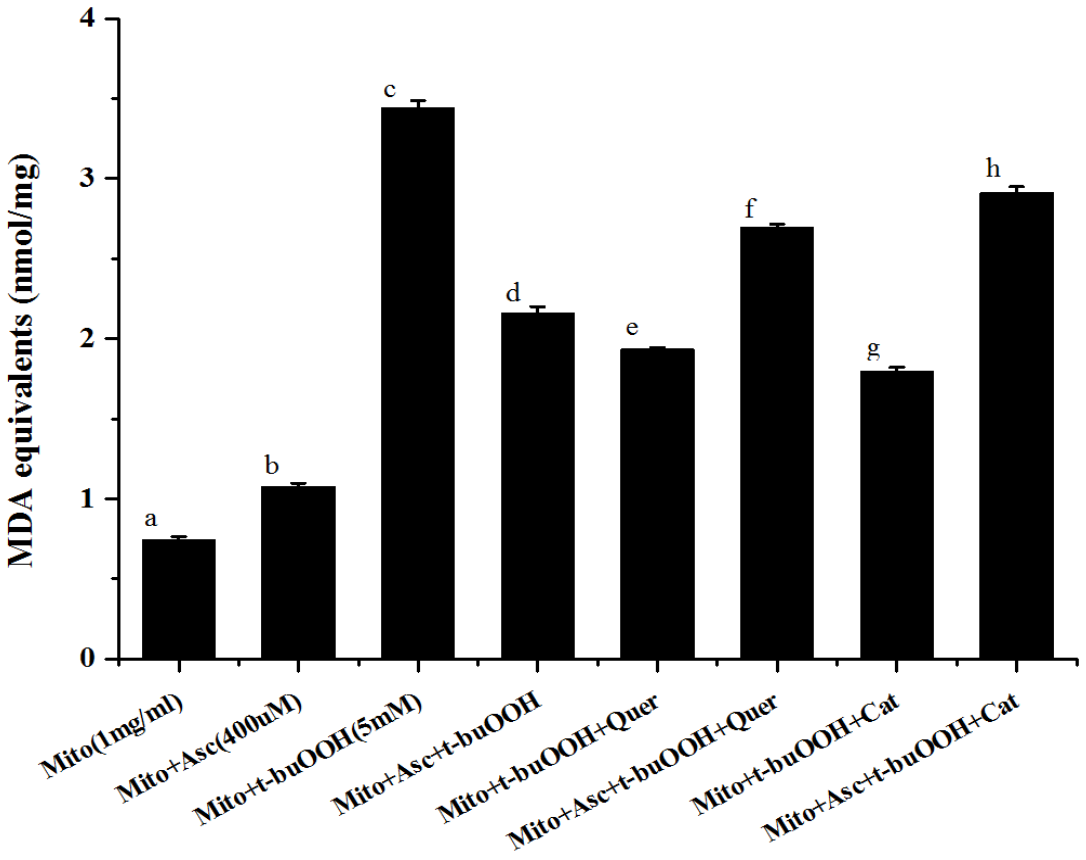


Figure 22. *Effects of quercetin, catechin and ascorbic acid on TBARS generation in mitochondria.* Mitochondria (1 mg/ml) were incubated with KPi buffer (pH 7.2, 50 mM) for one hour (t-buOOH 5 mM, Asc – ascorbic acid 400 µM, Quer- quercetin 50 µM; Cat- catechin 50 µM. [a-h] Different letters show significant differences by one-way ANOVA (post-hoc by Tukey's HSD) at $p<0.05$ (n=3 preparations, with 3 replicates per preparation). Bars represent means ± SEM.

4.3. Effects of ascorbate and flavonoids on α-tocopherol content and TBARS in rat liver mitochondria incubated with t-buOOH

Experiments were conducted to measure both TBARS and the α-tocopherol content in samples of mitochondria after incubation with t-buOOH. In the TBARS results (Figure 23), air incubation did not induce a statistical difference in mitochondrial oxidation level, but peroxide increased the TBARS formation significantly. In these experiments, only

catechin alone limited the oxidation significantly, although the inhibition by quercetin alone was close to significant ($p = 0.085$).

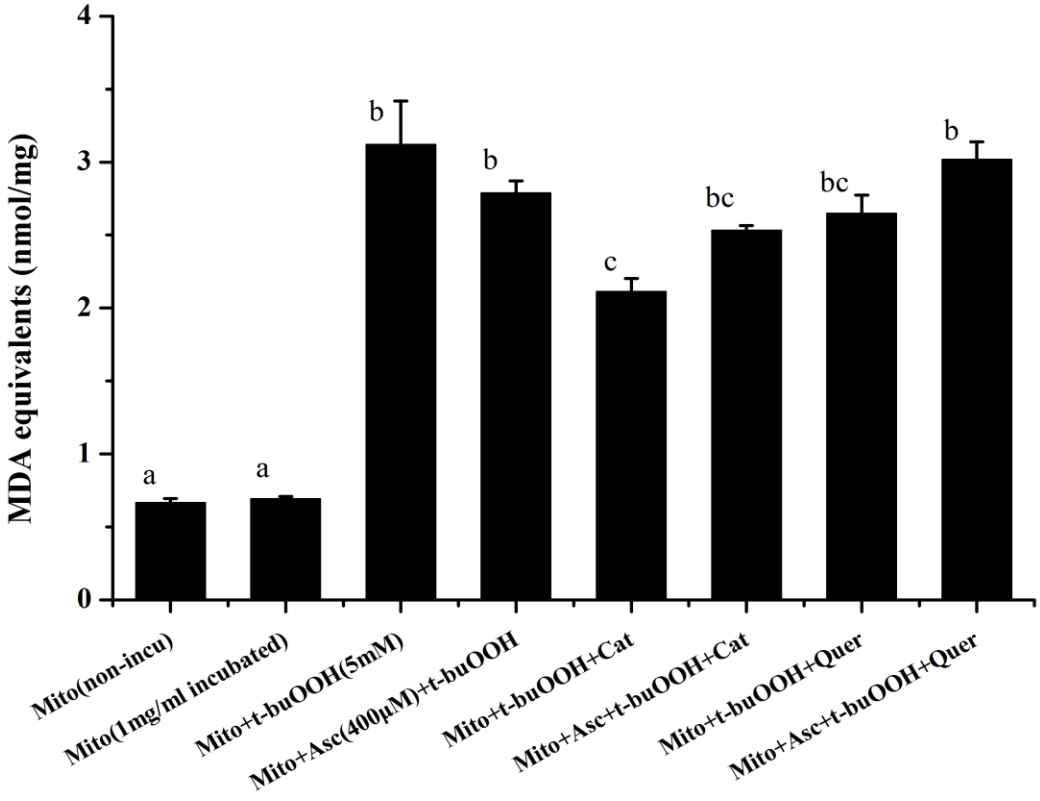


Figure 23. *Effects of ascorbic acid and flavonoids on t-buOOH-induced TBARS generation in rat liver mitochondria.* Mitochondria (1 mg/ml) were incubated with KPi buffer (pH 7.2, 50 mM) for one hour (t-buOOH 5 mM, Asc – ascorbic acid 400 μM, Quer – quercetin 50 μM, Cat - catechin, 50 μM. [abc] Different letters show significant differences by one-way ANOVA (post-hoc by Tukey's HSD) at $p<0.05$ (n=4 preparations with one measurement per preparation). Bars represent mean ± SEM.

The concentrations of α-tocopherol in mitochondria after different treatments were measured by HPLC-MS/MS (Figure 24), and a closer look at one of the samples could be found in Appendix 1. Catechin's involvement in the treatment did not affect the α-tocopherol level, which was contrary to the protection effect observed in the TBARS results. Quercetin was more involved in the regeneration of α-tocopherol. Its presence

alone in the treatment did not deliver significant protection against α-tocopherol loss, but if ascorbate was also present in the treatment, there was a significant interaction (verified by two way ANOVA, $p < 0.0001$), maintaining the α-tocopherol at 5 times higher than the incubated mitochondria control group. The α-tocopherol level in the interaction group was not statistically different from the non-incubated mitochondria control group, $p = 0.084$). The results therefore present a lack of consistency with the TBARS and vitamin E levels.

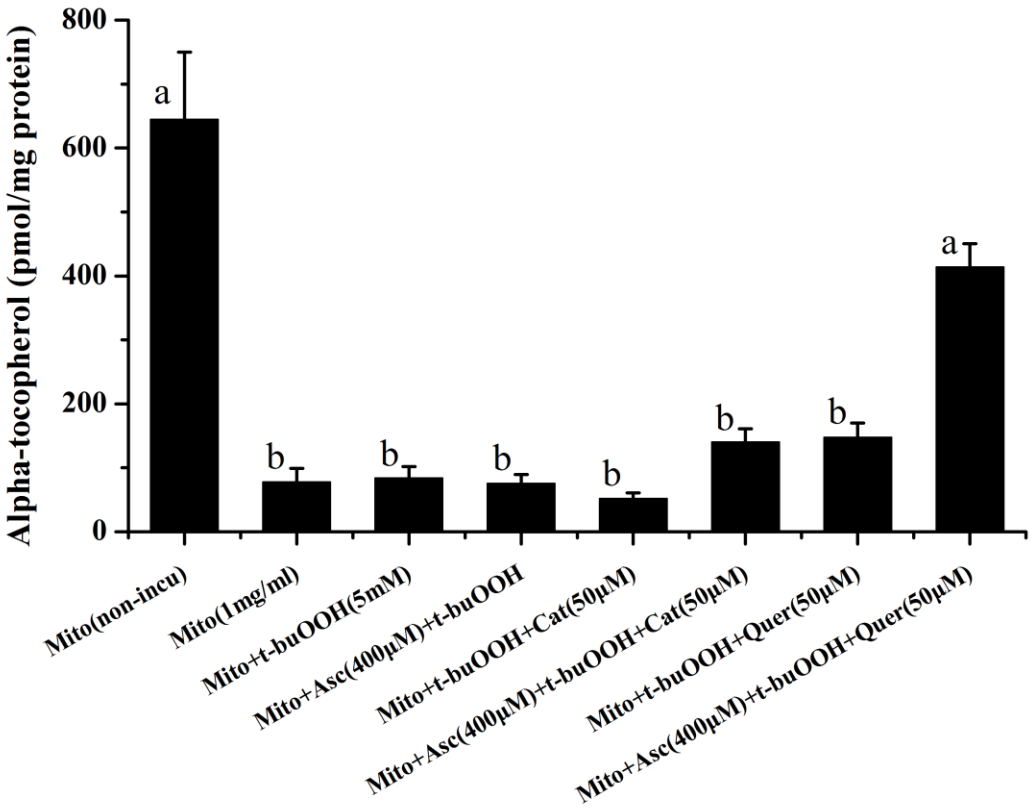


Figure 24. *Effects of ascorbic acid and flavonoids on α-tocopherol concentration in rat liver mitochondria after t-buOOH treatment.* [ab] Different letters show significant differences by one-way ANOVA (post-hoc by Tukey's HSD) at $p<0.05$. Bars represent mean ± SEM. Results are obtained from the same samples as in Figure 23.

5. DISCUSSION

5.1. Vitamin E protection from lipid peroxidation induced by vitamin C

In these experiments, we tested the ability of vitamin E to prevent a pro-oxidant effect of ascorbate on lipid peroxidation. Ascorbate has long been observed to provoke lipid peroxidation *in vitro* (17, 19-21), perhaps because of its ability to participate in the Fenton reaction. When ascorbate and free metal ions are both present in the environment, the ascorbate would reduce metal ions, which would in turn reduce hydroperoxides to generate free radicals (the Fenton reaction). By using the highest quality of water and buffer salts, we minimised the presence of metal ions in the buffer and explored whether vitamin C alone was able to induce pro-oxidant effects. In Figures 10 to 12, vitamin C showed its ability to induce TBARS generation in both liposomes (without and with vitamin E) and mitochondria. Vitamin E-integrated liposomes (0.0028mg/mg) had an increase of 16% in TBARS values after being treated with 200 μM ascorbate and 39% with 400 μM ascorbate. The higher concentration of ascorbate was able to bring the difference to a significant level. The same concentrations of ascorbate increased the TBARS by 17% and 35% in mitochondria, which were both statistically different from the control group. The mitochondria contain endogenous metals which may contribute to the Fenton reaction, while with liposomes there may have been traces of catalytic metals. Alternatively, pre-formed hydroperoxides in the liposomes and mitochondria may have been reduced by ascorbate to produce peroxyl radicals that propagate lipid peroxidation.

In 1999, Carr and Frei (29) reviewed the past *in vivo* studies on pro-oxidant effects of vitamin C and concluded that vitamin C does not act as a pro-oxidant. Among the 44 *in*

vivo studies they reviewed, 38 showed a reduction in various biomarkers, 14 showed no change and only 6 showed an increase in oxidative damage after supplementation with vitamin C. They suggested that the pro-oxidant effects were from errors or metal contamination during the experiment. In an earlier review in 1996, Halliwell discussed the connection between *in vitro* studies and the possibility *in vivo* of elevated oxidative stress biomarkers and pro-oxidant effects of ascorbate (17). From the results of our studies, although it was not an *in vivo* experiment on human subjects, there still was some evidence against Frei's theory that pro-oxidant effects are due to metal contamination (29). Both tested systems were maintained metal free, while the lipids were still oxidized by adding ascorbate. As a result, the oxidative stress induced by vitamin C in this research was different from the traditional pro-oxidant effects, which was performed by combination with metal ions. Another possibility raised by Chen (111) was that the adverse effects of vitamin C may be suppressed by vitamin E, indicating that the status of vitamin C as pro-oxidant or antioxidant depend on the concentration of vitamin E.

The current experiments to test the ability of vitamin E in protecting against the pro-oxidant activity of vitamin C were complicated by the very high levels of background oxidation in liposomes prepared in the absence of vitamin E. This oxidation occurred, presumably from sonication, despite efforts to decrease peroxidation by saturating the buffer with nitrogen. However it can be seen that 200 μM ascorbate induced TBARS formation to a level of 8.5 μM in liposomes without vitamin E (Figure 12), while in the presence of even a small amount of vitamin E the level of TBARS produced was only 0.8 μM (Figure 10). Also, in mitochondria, which contain vitamin E (Figure 11), the levels induced by 200-400 μM ascorbate were only 1.0-1.1 nmol/mg (0.8 μM in an equivalent

amount of lipid to the liposome experiments). It appears therefore that vitamin E provides at least partial protection to the membranes against the pro-oxidant effect of vitamin C. This protection would be consistent with the results of studies by Chen (111), who reported in rats that dietary vitamin E would ameliorate the oxidative stress induced by excessive vitamin C. Nevertheless, incubation with ascorbate also induced lipid peroxidation in mitochondria that had normal levels of vitamin E (Figure 11), showing that vitamin E does not provide complete protection.

5.2. Antioxidant and pro-oxidant effects of vitamin E

Comparing the TBARS levels of the control liposomes with and without vitamin E, the difference between them was almost 10 times. The liposomes without vitamin E suffered intensive initial oxidation, which could not be reduced back by adding any antioxidants or their combinations. On the other hand, even with the lowest integration amount of the vitamin E, it could prevent the initial oxidation and protect it against the peroxide. The experiments on liposomes with vitamin E are more biologically relevant since mitochondria are integrated with sufficient vitamin E in normal situations. In the aging process, the brain mitochondrial TBARS level of mice increased from 5.3 to 8.2 pmol/mg protein after 50 weeks (124) and in human Alzheimer's disease, the mitochondria TBARS level is also increased within this range (125). These results demonstrate that in the worst case where the brain mitochondria are seriously damaged by oxidative stress, the oxidation level is far less than our mitochondria studies (Figures 21-23, TBARS level around nmol/mg). Although the component of mitochondria in different organs might vary, it is still clear that the protection on lipid membrane from vitamin E is not

sufficient. It requires further protection from other antioxidants in order to maintain normal physiological conditions.

An interesting finding of the current studies with liposomes was a dose-dependent pro-oxidant effect of vitamin E that increased TBARS formation during air-incubation of liposomes. Vitamin E has also previously been reported to have pro-oxidant effects (126, 127). One possible explanation could be that the vitamin E radical, when it exists at higher concentrations in lipid membranes, may react with unsaturated lipids (LH) and lipid hydroperoxides (LOOH) to propagate lipid peroxidation (Figure 25) (126).

$$\alpha\text{-Toc}\cdot + \text{LH} \longrightarrow \alpha\text{-TocH} + \text{L}\cdot \qquad (1)$$

$$\alpha\text{-Toc}\cdot + \text{LOOH} \longrightarrow \alpha\text{-TocH} + \text{LOO}\cdot \qquad (2)$$

Figure 25. *Pro-oxidant mechanism of α-tocopherol (adapted from (126))*

Redox (reduction-oxidation) reactions are complicated processes involving electron transfer or change in oxidation state by a molecule, atom, or ion. Whether a compound acts as an oxidant or reductant is relative, and it can act as both with different rates with different reactants. The radical of α-tocopherol is able to reduce the lipid peroxyl radical into lipid hydroperoxide (Figure 6), but it can also oxidize free lipid and lipid hydroperoxide (Figure 25). With the presence of a stronger reductant, ascorbate in this case, the α-tocopherol radical can be consistently reduced and the redox balance is pushed forward to the reduction side. When stronger reductants or other radicals are not present, the α-tocopherol radical will react with the free lipid molecule, yielding lipid radical (reaction 1 in Figure 25 (126)), or react with lipid hydroperoxide, producing lipid

peroxyl radical (reaction 2 in Figure 25 (128)). The results in Figure 20 can be explained by this theory. With increasing amount of vitamin E integrated, the more competitive it is in rescuing free lipids (reducing lipid peroxyl radicals), and therefore more vitamin E radicals are formed. Due to limited exposure to strong oxidants in air incubation, the vitamin E radicals tend to deplete hydrogen atoms from free lipid molecules, resulting in higher TBARS formation.

5.3. Flavonoid protection from lipid peroxidation induced by vitamin C in the absence of vitamin E

The results in liposomes without vitamin E showed that, when quercetin was added into the solution without ascorbate it reduced the TBARS formation during air incubation, and together with ascorbate, quercetin was able to reduce the pro-oxidation effect of ascorbate to a significant level (Figure 12). The results indicate that quercetin can reverse the pro-oxidant effect of ascorbate, and together with ascorbate may rescue the liposome when vitamin E was insufficient in the membrane system. However, it might not occur in the normal situation as the mitochondria accumulate vitamin E and the level of vitamin E may preclude protection by quercetin. Therefore in situations of vitamin E deficiency, this effect of quercetin in protecting against pro-oxidant activities of ascorbate might be increasingly important. Moreover, incubation with ascorbate also induced lipid peroxidation in mitochondria that had normal levels of vitamin E (Figure 11).

5.4. Cooperation of flavonoids, vitamin C and vitamin E to inhibit lipid peroxidation

When a stronger oxidant (t-buOOH) was present in the environment, vitamin C acted differently in the two tested lipid membrane systems. In liposomes (Figures 15 to 19), further addition of ascorbate after peroxide did not decrease the TBARS generation, indicating that either ascorbate alone or in combination with vitamin E was not able to ameliorate the oxidative stress on liposomes. However, in mitochondria (Figures 21 to 23), ascorbate acted as radical scavenger and relieved the mitochondria from peroxide-induced oxidative stress. The opposite behavior of vitamin C between the two systems could originate from the different composition of membrane structures. With mitochondria, many bio-active molecules embedded in the double layer membrane could enhance the antioxidant properties (eg. DHA reductase may help re-reduce vitamin C, while glutathione and peroxidases may help with antioxidant activities (10, 11)). Therefore, the antioxidants were more efficiently used and connected to each other in preventing the oxidative stress.

Although multiple combinations of ascorbate, flavonoids and vitamin E at different concentrations were tested using the liposome model, no synergism was confirmed in these experiments. These results were against the initial hypothesis that the combination of the antioxidants was able to ameliorate the oxidation, indicating that vitamin E, ascorbate and flavonoids were not synergistically reacting with each other in reducing oxidative stress. In other research focusing on the synergism of these antioxidants, Dai et al. (2008) were able to identify the synergistic effects of ascorbate, α-tocopherol and polyphenols in other membrane system (SDS micelles with linoleic acid) (129). They

utilized kinetic studies and electron paramagnetic resonance spectroscopy to demonstrate that the antioxidant combination possessed synergistic antioxidative efficacy. This antioxidative efficacy was due to regeneration of α-tocopherol by polyphenols, which was subsequently regenerated by ascorbate. The reported synergism was observed by monitoring linoleic acid hydroperoxide formation over time and the consumption rate of the antioxidants, and the major tested polyphenols were catechin derivatives. The TBARS test, on the other hand, that we used in this research was a general test measuring reactive aldehydes (such as malondialdehyde) as the oxidation products of all polyunsaturated lipids. Three prevalent phospholipid molecules were selected to simulate the actual content in membrane systems. The antioxidants could be more reactive with simple fatty acids dispersed in micelles such as those used by Dai et al. (2008) than with phospholipid liposomes. Other differences in analytical techniques and target analytes could also contribute to this deviation in conclusions.

In order to compare the two lipid systems in this research, we need to also compare the vitamin E concentration. As stated previously (in section 4.2.4), the rough comparison of the lipid content in the liposomal and mitochondrial membrane systems are equivalent at 1 mg/ml (lipid concentration) liposomes and 2 mg/ml (protein concentration) of mitochondria (123). For vitamin E, 0.072 mg/mg (vitamin E/ total lipids) liposomal α-tocopherol equals 0.2 nmol/mg lipid while the mitochondrial α-tocopherol content is around 1.3 nmol/mg total lipids (calculated based on Figure 24 control group data). In those conditions, no synergism of various antioxidants has been observed and the protection of vitamin E seems to be the dominant factor in reducing the oxidation. However, when the vitamin E level was manipulated to 0.0014 mg/mg lipid in liposomes,

quercetin (but not catechin or cyanidin) showed an additive protective effect (Figure 19). This might be due to the vitamin E being too low in concentration to cope with the oxidative stress and ascorbate and quercetin helped regenerate vitamin E or helped reduce the peroxide directly in the solution or membrane. This could be the reason that adding both ascorbate and quercetin did not decrease the TBARS formation in vitamin E rich liposomes (Figures 16 and 17) while they decreased the level in vitamin E-barren ones (Figures 15).

Similar to the results on liposome with vitamin E integration, the administration of antioxidants to mitochondria failed to protect synergistically in TBARS tests. However, the flavonoids alone were able to protect the mitochondria from oxidation while the addition of ascorbate in the presence of flavonoid worsened the oxidation status (Figures 21 - 23).

5.5. Synergism of flavonoids and vitamin C in regenerating vitamin E

The amount of α-tocopherol measured in the mitochondria (614 pmol/mg protein in mitochondria without incubation) was one third of the previously reported data of 2.12 nmol/mg protein in rat liver mitochondria (121). The γ-tocopherol content, 0.055 nmol/mg protein, was also reported in mitochondria from the previous research (110), although it was below the limits of detection in the current measurements. There were some differences in extraction method, HPLC mobile phase and mass spectrometry settings between the two studies. However, mitochondria from rat liver were used in both studies, making the results comparable. In both studies, the α-tocopherol content was the major component of the vitamin E in the mitochondrial membrane. (It should be noted

that commercial rodent lab chows are generally supplemented with α-tocopherol, so the amount observed in the mitochondria may be more than would be observed naturally.)

Quercetin and ascorbate were not able to synergistically reduce the lipid oxidation status in mitochondria or liposomes; however, this antioxidant team interacted with each other in regenerating the mitochondrial α-tocopherol content. In Figure 24, either quercetin or ascorbate alone was not able to increase the α-tocopherol amount consumed by peroxide administration. Quercetin helped the α-tocopherol recovery, from 83.7 to 147.6 pmol/mg protein, although it wasn't statistically significant. When combining the two antioxidants, the increase of α-tocopherol level was not simply an additive effect. The α-tocopherol level was promoted to 413.8 pmol/mg protein, which was recovered to the equivalent level as the non-treatment group. However, the change in α-tocopherol level did not affect the TBARS formation, indicating that the α-tocopherol level might not correlate to the mitochondrial oxidation level or a more complicated mechanism was involved.

The major paradox in interpreting the results with mitochondria was the lack of correspondence between α-tocopherol concentration and TBARS formation. Being the major component of vitamin E in tissues, α-tocopherol would be expected to be negatively related to the oxidation status of the lipid membranes. However, comparing the results in Figure 23 and 24, from the same samples, no relationship was observed between TBARS and α-tocopherol. Three groups stood out against our hypothesis, the non-incubated group, catechin-treated group and quercetin+ascorbate-treated group. Comparing the non-incubated and incubated mitochondria, the α-tocopherol was consumed by 88% after one hour air incubation at 37^{o}C, while the TBARS formation only increased by 4%. The cause of the α-tocopherol loss during incubation in KPi buffer

is unknown. The administration of 50 μM catechin did not protect α-tocopherol compared with the positive control group (with peroxide), while it ameliorated the oxidative stress on the mitochondria indicated by a 31% decrease in TBARS formation. The ascorbate-quercetin combination group did not show any protection against TBARS formation. However, it replenished the α-tocopherol content in mitochondria by nearly 400% compared to the positive control group.

One possible explanation for this phenomenon was that 5 mM peroxide induced acute oxidation in lipid membranes, whose reaction rate was higher than the regeneration of α-tocopherol. In the beginning of the oxidation, the lipids were quickly oxidized and collaterally consumed a large amount of α-tocopherol in order to prevent this reaction. The α-tocopherol radicals regenerated later by ascorbate-quercetin could not reverse the TBARS formation within the lipid membranes. An extension of incubation time could examine this theory. Applying this hypothesis, the synergism comes slowly and could not save the mitochondria in the initial stage, while longer incubation time can induce more oxidative stress and the α-tocopherol cannot be regenerated in the groups other than the one receiving quercetin-ascorbate treatment.

As reported previously, regeneration of vitamin E by vitamin C was found both *in vitro* (104) and *in vivo* (107). Vitamin E was also reported to be spared by flavonoids (112). In this research, however, the individual antioxidant was not able to regenerate the α-tocopherol alone in the mitochondrial system. The combination of ascorbate and quercetin was the only efficient treatment in vitamin E regeneration, which was reported for the first time.

6. CONCLUSIONS

The conclusions drawn from the research can be summarized as follows:

- Ascorbate alone can induce a pro-oxidant effect in both the liposome and mitochondria systems, and the vitamin E content in both systems, although protective, does not completely prevent this event. For example, 200 μM and 400 μM ascorbate induced 16% and 39% elevation in TBARS formation in liposomes containing 0.0028 mg/mg vitamin E. The same concentrations of ascorbate increased the TBARS by 17% and 35% in mitochondria.

- The presence of quercetin inhibits the pro-oxidant effect of ascorbate in vitamin E-free liposomes. Quercetin at 50 μM was able to reduce the pro-oxidant effect of 200 μM ascorbate in the plain liposomes.

- The combination of ascorbate and quercetin can regenerate the α-tocopherol in the mitochondria while not decreasing the TBARS formation after one hour incubation at 37^{o}C, indicating that the α-tocopherol concentration was not correlated to the TBARS formation.

Overall, based on the research data, ascorbate has pro-oxidative effect on both systems, which can be suppressed by quercetin but not completely by vitamin E. Among the tested flavonoids, only quercetin can synergistically react with ascorbate to regenerate α-tocopherol while this interaction cannot rescue the mitochondria from oxidative damage.

7. FUTURE DIRECTIONS

One technical question that needs to be addressed is that of multiple MS peaks observed for α-tocopherol. Although several possibilities were tested, the multi-peak problem of α-tocopherol still persisted in the results. Also, in future studies the combined concentration of vitamin E and its metabolic products might better serve as the indicator of the oxidative stress.

The pro-oxidant effects of vitamin C and E were not completely explored in the current research. The mechanism of pro-oxidant effects without free metal ions is unknown. Experiments with metal chelators may help identify any involvement of trace metals. Also, the ability of flavonoids to inhibit the pro-oxidant effect of ascorbate on mitochondria was not tested in the current studies.

Another interesting line to follow is the relationship between the vitamin E concentration within the mitochondrial membranes and the oxidative status. The current results with liposomes indicate that they are correlated positively to each other, while it is commonly believed that the oxidative stress should decrease as the antioxidant concentration increases.

As for the synergism of the different antioxidants, we tried the combinations of them in different concentrations while the exposure time was not well explored. The future study can focus on whether increasing the exposure time to the peroxide would reveal the ability of antioxidants to cooperate with each other by allowing time to further deplete vitamin E.

Finally *in vivo* studies can be another option for future directions. It will be important to determine the extent to which the interactions occur *in vivo*.

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APPENDIX 1

1. Multiple peaks in α-tocopherol

The specific ionization and breaking pattern of each compound should generate a single signal, and the interaction between compounds and the HPLC column should be consistent throughout the analysis. However, in reality, there are many factors that can influence the specificity of the analysis. In analyzing the α-tocopherol content, there are three separate HPLC peaks with the characteristic parameters for α-tocopherol structure (left part in Figure 1AP). Several possible reasons can be applied to this phenomenon.

1, Alpha-tocopherol can form a stable radical in the membranes with 1Da difference in molecular weight, and by forming the radical, the retention time is also shifted. This theory has been tested by adding a radical scavenger (2% BHT) into the sample. However, the multiple peaks remained in the results.

2, Instead of capturing the specific daughter ion(s), the tandem mass spectrometry analysis (MS^2) accumulates all the collision fragments and establishes a detailed picture of these fragments and their proportion. In comparing the MS^2 results of the three peaks from the chromatogram, their fragments were different and so were their proportions. This indicated that the three portions of the samples were collided into different fragments, and *ergo* did not share the same chemical structure. The MS^2 results of peak B in Figure 2AP showed three major fragments after collision, which were consistent with the three major fragments of standard α-tocopherol. The similarity confirmed that peak B was α-tocopherol extracted from mitochondria, in addition to the identical retention time. One of the fragments with mass over charge ratio of 239.1 is unique, and it can be

considered as the daughter ion to evaluate in pursuit of higher specificity of α-tocopherol detection in future analyses.

3, It has been reported that when α-tocopherol experiences intensive exposure to radicals, including peroxyl radicals, it is likely to be oxidized to α-tocopherol-5,6-quinone (tocored) (a). Tocored has a small difference in molecular weight compared to α-tocopherol (0.04 Da). Assuming that its daughter ion has the dimethyl benzoquinone structure with a single charge (Figure 2AP), the mass over charge ratio for this molecule and its counterpart from α-tocopherol would have similar molecular weights (differing by 0.04 Da), making them indistinguishable even using MS. The structural modification will lead to a shorter retention time as hydrophobicity increases, resulting in the earlier signal. This assumption is consistent with oxidation products described in research from Nagata et al (a), although they did not apply the collision cell in the mass spectrometry. However, this hypothesis suffers the problem of low tocored formation, which is more likely to be the product of γ-tocopherol instead of α-tocopherol.

4, Another possibility is that the enantiomers of α-tocopherol are separated by HPLC and generating signals at different time points. The possibility that the rat tissues contain different enantiomers is strengthened by the common supplementation of commercial rodent chows with α-tocopherol, which may be a racemic mixture (all-rac). In order to testify this hypothesis, we can test whether all-rac α-tocopherol as a standard would generate the same 3-peak result.

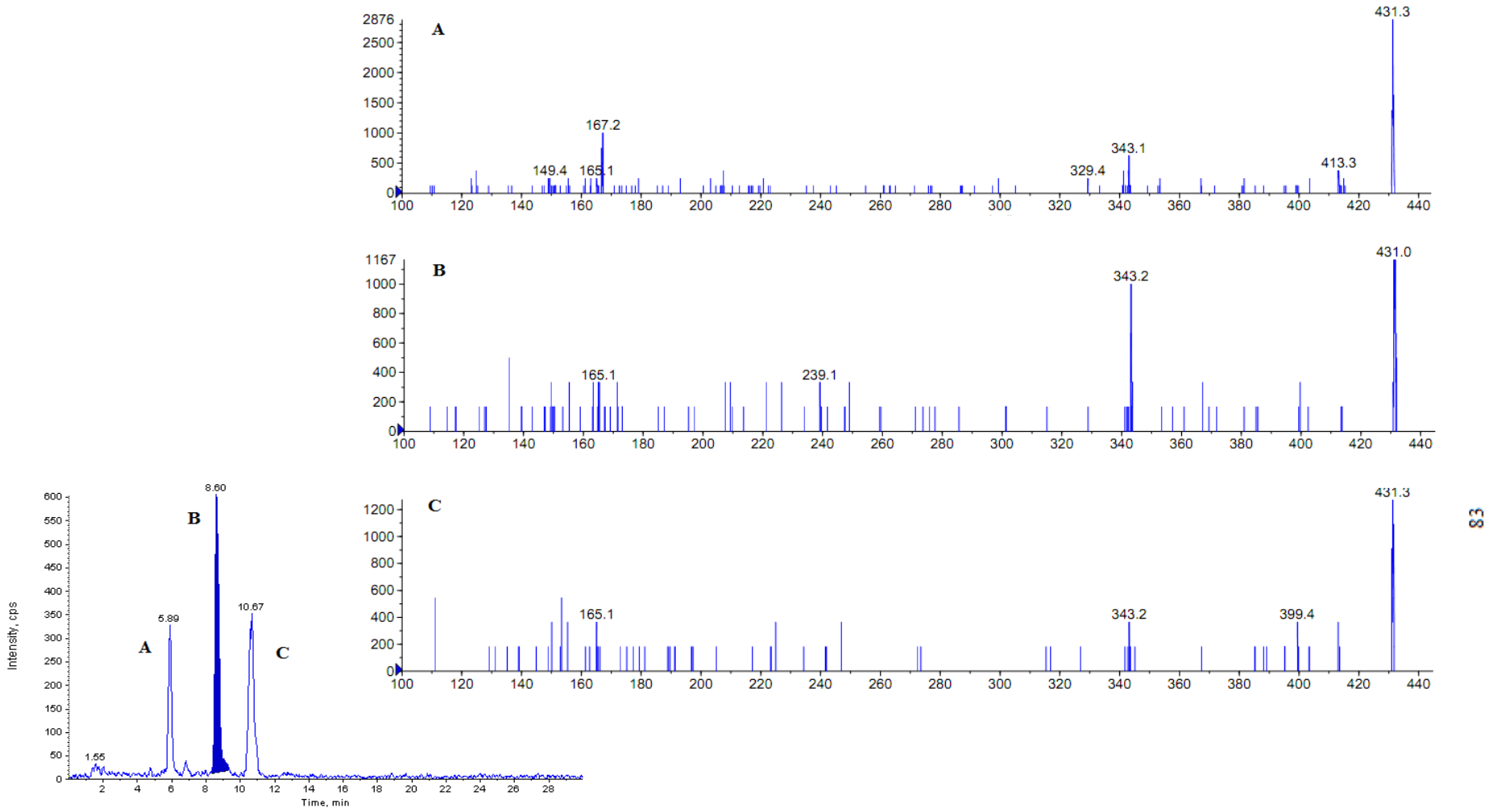

Figure 1AP. *The bottom chromatogram is MS1 analysis of the mitochondria extract while the other 3 stacked graphs are MS2 analysis on the three individual peaks. The highlighted peak is the target compound, as determined by comparison with the standards.*

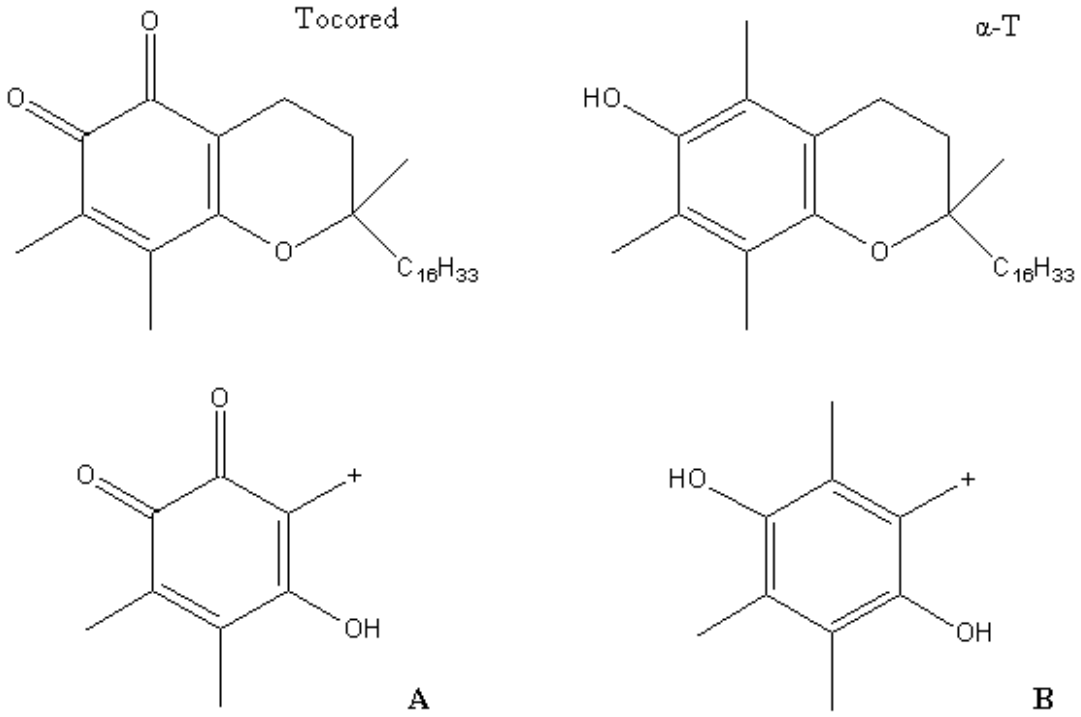

Figure 2AP. *Tocored (A) and α-tocopherol (B) with their possible daughter ions*

2. Gamma-tocopherol content determination using Mass spectrometry

The HPLC parameters were the same as alpha-tocopherol and internal standard. The individualized MS parameters were as follow: corona discharge current, 4.0 uA; source temperature, 400 ℃; collision-activated collision gas (nitrogen) setting, 7 (arbitrary units); curtain gas (nitrogen) setting, 6; and nebulizer gas (air) setting, 10. MRM measurements with the transitions m/z+ 417.3/151.1 for γ-T (b). The Lower Limit of Quantification (LLOQ) of γ-tocopherol was determined as 100 pM, at which concentration yielded peak heights were 10 times higher than the noise baseline. We also

established the standard curve for γ-tocopherol from 100 pM to 250 pM, through which the γ-tocopherol concentration and the corresponding signal was linear. However, when testing the extractant from mitochondria, the results was not greater than the LLOQ, therefore this part of the result was not included in the thesis.

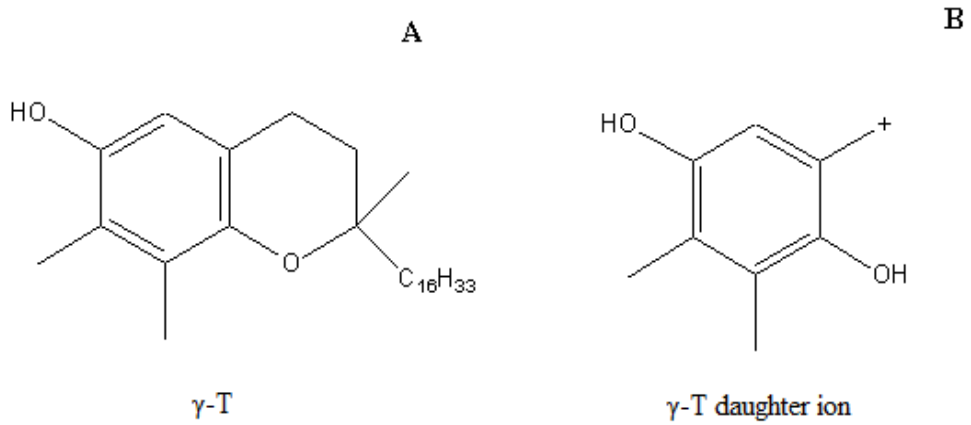

Figure 3AP. *The molecular structure of γ-tocopherol (A) and its daughter ions (B). Mass over charge ratio for B is 151.1.*

Appendix references

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